Heterogeneity of $GABA_A$ Receptors and Interneurons in the Amygdala

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Zusammenfassung

Die Amygdala, eine mandelförmige Gruppe von Kernen tief im Schläfenlappen, spielt eine wichtige Rolle bei Emotionen und damit verbundenen Lern- und Gedächtnisvorgängen. Der basolaterale und der zentrale Kern der Amygdala sind dabei von besonderer Wichtigkeit: sie werten und belegen einen eintreffenden sensorischen Reiz mit einer bestimmten emotionalen Bedeutung und lösen dann das entsprechende Verhalten aus. Wesentlich für den korrekten Ablauf dieses Assoziations-Prozesses ist das GABAerge inhibitorische System. Blockiert man es nämlich pharmakologisch, führt dies zu unkontrollierten, panikartigen Zuständen – auch ohne den entsprechenden Reiz löst die Amygdala nun Angst- oder Furchtverhalten aus. Trotz der zentralen Bedeutung des inhibitorischen Systems ist erstaunlich wenig über dessen Schlüsselkomponenten, den Interneuronen und $GABA_A$-Rezeptoren, bekannt.

Im ersten Teil dieser Arbeit wurde die Verteilung verschiedener $GABA_A$-Rezeptoren (unterschiedlich im Hinblick auf die α-Untereinheit) sowie ihr relativer Anteil an inhibitorischen Strömen mit immunohistochemischen und elektrophysiologischen Mitteln untersucht. Verwendet wurden Knock-in-Mäuse, in denen bestimmte $GABA_A$-Rezeptoren aufgrund einer Punkt-Mutation nicht mehr auf Diazepam, das am unveränderten Rezeptor zu einer charakteristischen Erhöhung des GABA-Stroms führt, reagieren. Wir fanden eine klare Kern-spezifische Verteilung vor, wobei der Grossteil der inhibitorischen Ströme im basolateralen sowie im zentralen Kern von α2-$GABA_A$-Rezeptoren vermittelt wurde. Erstaunlicherweise liessen sich keine α1-$GABA_A$–Rezeptor-vermittelte Ströme im zentralen Kern registrieren, während die α3-Untereinheit nur minimal in beiden Kernen zur Inhibition beitrug. Diese funktionellen Daten wurden mittels immunohistochemischen Färbungen bestätigt. Sie untermauern in früheren Studien gewonnene Resultate, wonach die α2-$GABA_A$-Rezeptoren die anxiolytische Wirkung von Diazepam vermitteln; ein Effekt, der über Schaltkreise in der Amygdala abläuft. Im Hippocampus formen Interneurone, die den Cannabinoid Rezeptor 1 (CB1) auf ihren Endigungen tragen, bevorzugt Synapsen mit α2-$GABA_A$-Rezeptoren. Wir konnten zeigen, dass dies nicht für Interneurone in der Amygdala gilt. Zumindest im basolateralen Kern trägt der grössere Teil der CB1-Synapsen α1-enthaltende $GABA_A$-Rezeptoren.

Im zweiten Teil dieser Arbeit haben wir einen bestimmten Typus Interneurone charakterisiert, die sogenannten parakapsulären Zellen. In Mäusen, die ein grün-fluoreszierendes Protein in allen Interneuronen exprimieren, lassen sich diese Zellen leicht erkennen: Sie formen grossflächige Cluster entlang der lateralen und medialen Grenze des basolateralen Kerns. Ihre Nähe zu corticalen Afferenzen liess vermuten, dass sie von diesen innerviert werden und Zellen in der Amygdala hemmen. Tatsächlich konnten wir nachweisen, dass die parakapsulären Zellen des lateralen Clusters cortical aktiviert werden und inhibitorische Verbindungen zu Pyramidenzellen im basolateralen Kern bilden. Bereits früher wurde für die Zellen des medialen Clusters gezeigt, dass sie in den zentralen Kern projizieren. Demnach, so unsere Schlussfolgerung, bilden die parakapsulären Zellen als Ganzes ein feedforward-Netzwerk, das unter corticaler Kontrolle steht und den Informationsfluss in die und aus der Amygdala steuert.

Dieses Netzwerk wird von Dopamin moduliert. Durch Aktivierung von D1-Dopamin-Rezeptoren auf den parakapsulären Zellen werden G-Protein-abhängige Kalium-Kanäle geöffnet, worauf die Zellen hyperpolarisieren und an elektrischer Erregbarkeit verlieren – ein einzigartiger Effekt in der Amygdala, da Pyramidenzellen und andere Interneuronen via D1-Rezeptoren-Aktivierung depolarisiert und damit erregbarer werden. Zudem konnten wir zeigen, dass Dopamin zusätzlich über einen präsynaptischen Effekt die Freisetzung von GABA unterdrückt und teilweise auch den exzitatorischen Input reduziert. Insgesamt vermindert Dopamin also die inhibitorische Wirkung der parakapsulären Zellen und führt damit zu einer Disinhibition der Amygdala.

Summary

The amygdala, an almond-shaped group of nuclei at the heart of the telencephalon, plays a crucial role in emotion and associated cognitive functions such as learning and memory. The basolateral and the central nucleus of the amygdala are particularly critical for producing emotionally appropriate behaviour in response to sensory stimuli. For this process, intensively studied in fear conditioning, an intact GABAergic inhibitory system seems to be essential. This is evidenced by the fact that selective blocking of inhibition in the amygdala results in an uncontrolled anxiety-like state. Despite their importance, functional data on interneurons and $GABA_A$ receptors within these nuclei are scant, mostly because inhibitory cells in this part of the brain possess an inconspicuous morphology and are thus difficult to identify.

Using knock-in mice, in which a point-mutation renders specific $GABA_A$ receptors diazepam-insensitive, the contribution of different $GABA_A$ receptors (in terms of the α-subunit) to inhibitory currents was investigated with electrophysiological techniques. We detected a clear nucleus-specific distribution with α2-$GABA_A$ receptors carrying the bulk of inhibitory currents in the basolateral as well as the central nucleus. Surprisingly, the α1-subunit was completely absent from the central nucleus, while the α3-subunit contributed only modestly to inhibition in both nuclei. These functional results, confirmed by immunohistochemical staining, are in line with recent studies from our institute, in which α2-$GABA_A$ receptors were found to mediate the anxiolytic action of diazepam. In addition, we detected that inhibitory terminals carrying cannabinoid receptor 1 (CB1) targeted predominantly α1-subunit containing $GABA_A$ receptors, which is in contrast to the situation in the hippocampus.

The second part of this study focussed on a specific type of amygdala interneuron, the so-called paracapsular cells. In mice that express green fluorescent protein in all interneurons, these small cells could be easily identified, forming prominent clusters along the lateral and the medial border of the basolateral complex. Their location in close proximity to afferent fibre bundles originating in the cortex makes them prime candidates for mediating cortical inhibition in the amygdala. Indeed, we found that cells of the lateral cluster provide cortical feedforward inhibition onto projection cells of the basolateral nucleus, similar to those of the medial cluster that target cells of the central

nucleus. These findings support the notion that the paracapsular cell system as a whole can be viewed as an inhibitory feedforward network that controls the flow of information into as well as out of the amygdala. Dopamine modulates this inhibitory gate: The increase of a G-protein dependent potassium conductance by D1 receptor activation hyperpolarizes paracapsular cells and reduces their excitability – a unique effect, as projection cells as well as interneurons within the basolateral nucleus depolarize in response to dopamine and D1 receptor agonists. Furthermore, dopamine suppresses the presynaptic release of GABA from paracapsular cells and partly reduces their excitatory input. Taken together, the inhibitory output of the paracapsular cells system is greatly diminished under dopamine, leading most likely to a disinhibition of the amygdala. These findings complement recent *in vivo* studies, in which dopamine (via D1 receptor activation) was shown to attenuate cortical suppression of the amygdala, thereby facilitating sensory-driven affective responses.

Considering the specific pharmacology of the behavioural effects together with the fact that the paracapsular cells are the only amygdala interneurons that are inactivated by D1 receptor activation, we conclude that the paracapsular cells system represents the neural substrate that mediates prefrontal cortical control over the amygdala.

1 General Introduction

1.1. The Amygdala

The amygdala (from greek amygdale = almond), first described 1819 by the German anatomist Burdach, comprises a complex of several nuclei located deep in the temporal lobe of the brain. This evolutionarily old structure is of particular interest because of its central role in decoding and producing emotions, related especially to stimuli that are threatening to the organism.

Earliest evidence for its function stems from experiments, in which monkeys with bilateral temporal lobectomy showed a drastic decrease in fear and in emotions in general (Kluver and Bucy, 1937; Kluver and Bucy, 1939). Consistently, a pattern of "flattened affect" is commonly described in clinical case reports of human patients with amygdala damage (Aggleton, 1992). Further, electrical stimulation and epilepsy studies, both focussing on the amygdala, emphasized a role for this structure in the processing of negative emotions, as subjects reported unanimously that the predominant feeling triggered by electric discharges was anxiety or fright (Aggleton, 1992). Moreover, similar stimulation experiments conducted in several different species of mammals repeatedly generated the same well-known defense reactions (inhibition, flight, defensive attack), thus indicating the evolutionary advantages of such an automatic reaction evoked by fear. Interestingly, lower-voltage stimulation produced primarily "alerting" and "searching" behaviour in rats, which led to the interpretation that typical "fearful" behaviours might be elaborated from these more attentional behaviours (Davis and Whalen, 2001).

Yet the amygdala is not simply a "generator" of fear. It is also important for the recognition and interpretation of emotions (especially negative ones) conveyed by facial expressions (Adolphs et al., 1994; 2005; Morris et al., 1997; Tovee, 1995). Indeed, in animal species depending on their auditory rather than their visual sense, growls, screeches and other negative signs lose their meaning after surgical removal of the amygdala and become incomprehensible as afferent cues. Thus, in a broader definition, the amygdala is essential for linking salient sensory stimuli with affective outcomes and

initiating emotionally appropriate behaviour. Moreover, the amygdala forms implicit (non-conscious) emotional memories associated with fear (Fendt and Fanselow, 1999).

Although the involvement of the amygdala in positive affects has long been outshone by its prominent role in fear, more recent studies point to an equally important function in non-threat-related behaviour, such as specific kinds of stimulus-reward-learning. For example, monkeys with lesions of the temporal pole including the amygdala poorly learn new stimulus-reward associations, suggesting an altered emotional response to reward (Baxter and Murray, 2002). In rats, excitotoxic axon-sparing lesions of the amygdala diminish the capacity of stimuli associated with reward - such as access to female rats for male rats - to motivate behaviour (Aggleton, 2000). Furthermore, in humans the amygdala also responds to happy faces, though this activation occurs unilaterally (only the amygdala in the left hemisphere, commonly associated with positive emotions, is activated) and in terms of intensity varies across people, presumably as a function of extraversion (Canli et al., 2002). Other studies document an involvement of the amygdala in social behaviour (Ferguson et al., 2000) and in emotional modulation of memory (McGaugh, 2004).

Essential for the understanding of our study is the fact that the amygdala is critically involved in imbuing sensory stimuli with the appropriate emotional significance - an association that can be memorized and impacts upon aversive as well as appetitive behaviour. Though expression of such emotional associations is doubtlessly important for survival, it is not always appropriate and may even be disadvantageous in certain situations. For example, a “fight or flight” response with its associated hormonal, autonomic, and behavioural changes is inappropriate, even pathological in situations in which danger is unlikely. Likewise, uncontrolled response reaction to appetitive stimuli may underlie addictive behaviour. Therefore, the expression of emotional associations stored in the amygdala must be tightly controlled and gated by contextual cues, conscious reasoning (Bouton and Sunsay, 2001; Hariri et al., 2000) and, on a network level, by inhibitory mechanisms. As emphasized by LeDoux, failure in these inhibitory mechanisms can lead to “overexpression” of conditioned associations, which could appear as pathological states such as anxiety disorders (LeDoux, 1996; Shekhar et al., 1999) and drug-seeking behaviour (Quirk and Gehlert, 2003).

Several lines of evidence further support the importance of the inhibitory system for the proper functioning of the amygdala:

1) Blocking inhibition by microinjection of $GABA_A$ receptor antagonists into the amygdala of rats results in an anxiety-like state with an increase in heart rate and blood pressure (Sanders and Shekhar, 1995).
2) Drugs that induce anxiolysis such as benzodiazepines suppress the firing rate of neurons in the amygdala (Chou and Wang, 1977) by potentiating GABAergic inhibitory transmission.
3) The spontaneous firing rates of neurons in those amygdala nuclei most important for the processing of fear are among of the lowest in the brain, suggesting pronounced inhibition (Gaudreau and Pare, 1996; Pare and Gaudreau, 1996).

Given the pathophysiological implications of an impaired inhibitory network in the amygdala, one would expect a significant research interest in this topic. Yet physiological data on inhibitory mechanisms in the amygdala are scant mostly due to the principal difficulty of identifying the two key elements in this circuit: the interneurons and the different types of $GABA_A$ receptors.

Transgenic mice available to our laboratory, in which either interneurons are marked with a green fluorescent protein or a mutation is introduced in a specific alpha subunit of the $GABA_A$ receptor, rendering the receptor insensitive to benzodiazepines, enable a new approach to this subject.

1.2. Anatomy of the Amygdaloid Complex

The amygdaloid complex is structurally diverse and consists according to different authors of up to 13 nuclei, each of which can be further divided into several subnuclei depending on their cytoarchitecture, histochemistry and interconnections.

In the rat, the species in which most functional studies are carried out, the nuclei are classically divided into four groups (Price, 1987) (see Fig. 1):

1) The deep or *basolateral (BLA)* group, including the lateral nucleus (LA), the basal nucleus (B) (which is often further subdivided into a basolateral and a basomedial part), and the accessory basal nucleus (AB). The basolateral group is the largest division of the amygdala and can be easily identified in a coronal section because it is outlined by two prominent fibre bundles, the external capsule on the lateral side and the intermediate capsule on the medial side, forming an upside-down V.

2) The superficial or *cortical-like* group, including the cortical nuclei, the nucleus of the lateral olfactory tract (NLOT), the bed nucleus of the accessory olfactory tract (BAOT) and the periamygdaloid cortex (PAC); although these structures are called nuclei, many display cortical characteristics like a layered structure in accordance with their location at the surface of the brain.
3) The *centromedial* group, composed of the amygdaloid part of the bed nucleus of stria terminalis (BNST) and the medial (M) and central nuclei (CeA). The CeA is further subdivided into a lateral (CeL) and a medial (CeM) part.
4) A separate set of nuclei comprises the amygdalohippocampal area and the intercalated nuclei. The latter, however, are composed of several clusters and it is therefore unclear whether they indeed form a nucleus or at least partly belong to the BLA complex. Price (1987) solely marked the largest group, the rostrally located main intercalated cell masses (I), in his brain schematic.

As evident from this list of heterogenous nuclei, the amygdala is far from being an anatomical entity – it is in fact a rather arbitrarily assembled structure that can be principally divided into two entirely different parts: the central and medial nuclei constitute a phylogenetically older component whereas the basal, lateral and cortical nuclei, which have presumably emerged by infolding and proliferation of the piriform lobe, make up the phylogenetically more recent part (Swanson, 2003). Consistent with its embryological origin, the central and medial nuclei are organized similarly to the dorsally situated striatopallidum while the BLA complex shares strong similarities with the cortex in terms of cell content, cell morphologies and neurotransmitters.

Of all amygdala groups, the BLA complex is by far the best-characterized and most intriguing due to its central role in fear learning and fear conditioning. Consequently, investigation of the inhibitory system will focus on this nucleus; likewise connectivity as laid out in the next chapter will spotlight the BLA complex.

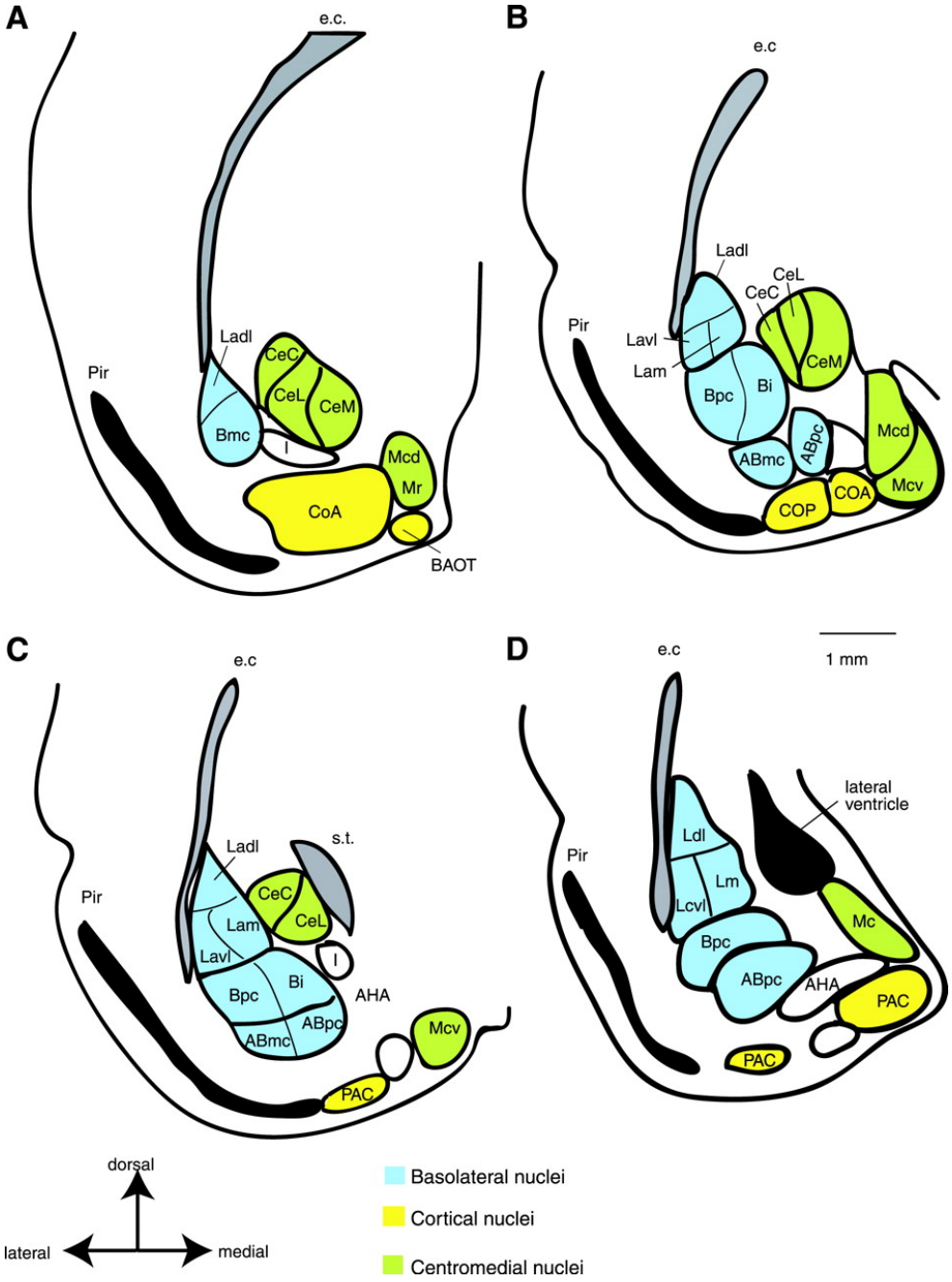


Figure 1 Nuclei of the rat amygdaloid complex. Coronal sections are drawn from rostral (*A*) to caudal (*D*). The different nuclei are divided into three main groups with the intercalated cells (I) and the amygdalahippocampal area constituting a separate set of nuclei depicted in white. Areas in blue form part of the basolateral group, areas in yellow are the cortical group, and areas in green form the centromedial group. ABmc, accessory basal magnocellular subdivision; ABpc, accessory basal parvicellular subdivision; Bpc, basal nucleus magnocellular subdivision; e.c., external capsule; Ladl, lateral amygdala medial subdivision; Lam, lateral amygdala medial subdivision; Lavl, lateral amygdala ventrolateral subdivision; Mcd, medial amygdala dorsal subdivision; Mcv, medial amygdala ventral subdivision; Mr, medial amygdala rostral subdivision; Pir, piriform cortex; s.t., stria terminalis. (Sah et al., 2003)

1.3. Connectivity of the Amygdala

The amygdala maintains connections to virtually all structures in the brain with most neural pathways entering the amygdala being paired with reciprocal pathways (Aggleton, 2000). Zooming in on the BLA complex limits these numerous connections to roughly ten, which are sketched in light of their function in emotional processes in the following section. Subsequently, for a more detailed understanding of amygdala connectivity, the concept of pavlovian fear conditioning will be introduced and thereby interamygdaloid connections will be presented along with the main in- and output pathways of the amygdala.

Afferents

Since the amygdala controls several of the alarm circuits of the body, many sensory inputs converge here to inform of potential danger in the environment:

- This sensory information comes either directly from the *sensory thalamus* or from the various *sensory cortices*.
- Likewise, the *hippocampus* sends strong projections to the amygdala. Since this structure is involved in storing and retrieving explicit memories, its connections to the amygdala may be the origin of strong emotions triggered by particular memories. Furthermore, the hippocampus specializes in processing sets of stimuli (as opposed to individual stimuli) that reproduce the context of a situation; because of its close connection to the amygdala the entire context associated with a traumatic event can provoke anxiety.
- The *prefrontal cortex* seems to be involved in the final phase of confrontation where, after the initial automatic emotional reaction, we are forced to react rationally and choose the course of action that can best get us out of danger. Thus, this superior mental structure enables a voluntarily planned response suited to the situation, making it possible to exercise a certain conscious control over anxiety. Among the cortical connections, those originating in the *medial prefrontal cortex* play a distinct role. These connections appear to be involved in the process of fear extinction, where a stimulus that triggers a conditioned fear gradually loses this effect if it is repeatedly presented to the subject without the associated unconditioned nociceptive stimulus.

- Notably, the amygdala receives particularly dense monoaminergic innervation (dopamine, noradrenaline, serotonin), which modulates or fine-tunes amygdala-mediated behaviour

Efferents

Because emotions profoundly influence many functions of the central nervous system, the amygdala sends efferent connections to a wide array of structures.

- Basic vegetative functions such as the regulation of heart rate or body posture are controlled by efferents of the amygdala to the *hypothalamus* and *brainstem*.
- Connections between the BLA and *striatum* mediate stereotypical motor reactions and voluntary movement such as escape and avoidance.
- Efferents to the cerebral *cortex* guide attentional processes and steer cognitive function in emotion. In particular, the CeA has a well-described role in modulating attentional processing via outputs through basal forebrain cholinergic neurons to cortical regions.
- Efferents to the dentate gyrus, the input area of the hippocampus, are thought to underlie emotionally charged memories, which are often more vivid and longer lasting than those of neutral events.

1.3.1. Fear conditioning

Experimentally, fear conditioning consists of pairing of a noxious unconditioned stimulus (US) with a neutral conditioned stimulus (CS), with the result that a fearful state can be produced in an animal simply by presenting the previously neutral stimulus. The CS can be an auditory, visual, olfactory, or tactile stimulus; most commonly used are light or a tone. It is presented to the animal and then followed by an electrical shock or a loud noise, the US. After a few tone-shock pairings (one is often sufficient), responses that typically occur in the presence of danger are elicited by the tone (Fig. 2). Examples of such defensive responses include freezing, autonomic and endocrine responses, as well as alterations in pain sensitivity and reflex expression.

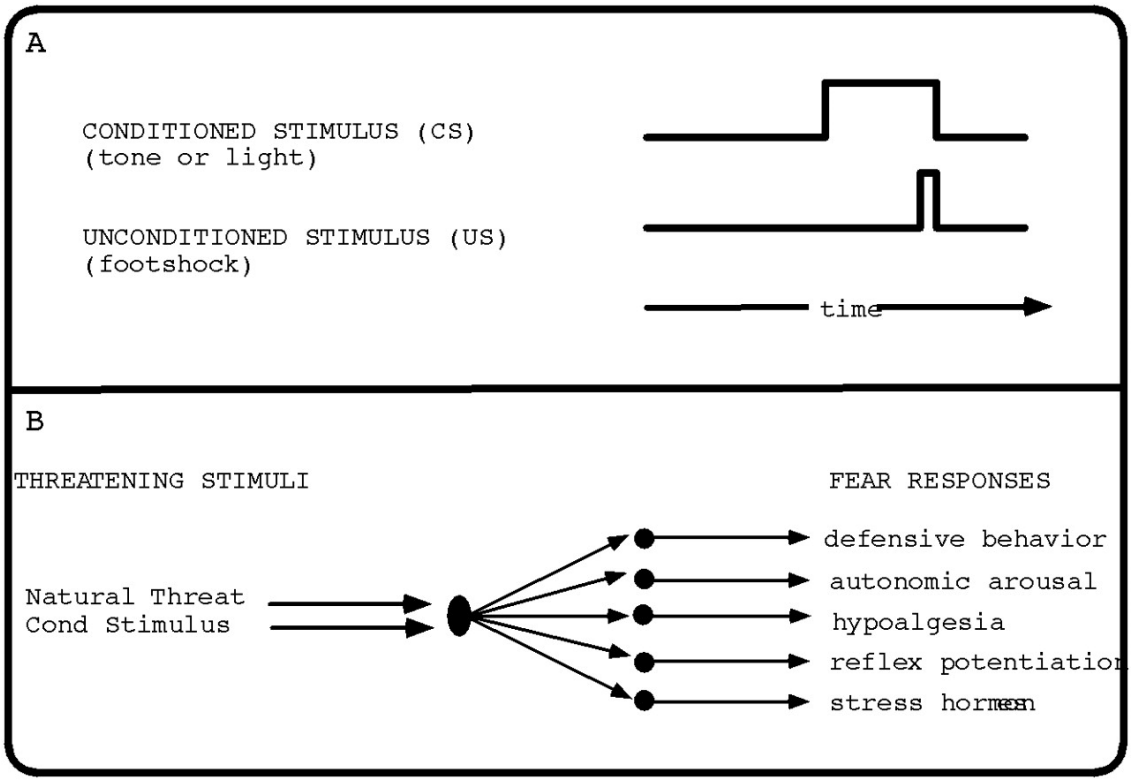


Figure 2 Fear conditioning involves the presentation of a noxious unconditioned stimulus, typically footshock, at the end of the occurrence of a relatively neutral conditioned stimulus (CS), such as a light or tone (*top*). After conditioning, the CS elicits a wide range of behavioral and physiological responses that characteristically occur when an animal encounters a threatening or fear-arousing stimulus (*bottom*). Thus, a rat that has been fear conditioned will express the same responses to a CS as to a natural threat (i.e. a cat). (LeDoux, 1996)

1.3.2. The Neuroanatomy Underlying Fear Conditioning

Studies over many years have clearly established that within the amygdala the BLA complex and the CeA play key roles in the acquisition and expression of fear-related behaviours (Goosens and Maren, 2001; Maren, 2000; Quirk et al., 1995). Although controversy persists (Cahill et al., 1999) it is widely believed that sensory input reaches the BLA complex via the LA nucleus that is considered to be the site where the CS-US association takes place (Blair et al., 2001; Maren et al., 2001). According to the current model, convergence of CS and US input increases the efficacy of synapses conveying information about the CS to the LA nucleus (LeDoux, 2000; Walker and Davis, 2000). As a result, subsequent presentations of the CS alone evoke larger responses in the LA (Pare and Collins, 2000; Quirk et al., 1995; Repa et al., 2001). The LA, in turn, evokes conditioned fear responses via its projections to the CeA (LeDoux et al., 1988; Maren and Quirk, 2004), which is the main source of amygdala outputs to brain stem and hypothalamic sites that produce fear responses (Bellgowan and Helmstetter, 1996; Davis and Whalen, 2001). The pathways involved in this model of fear conditioning are outlined in Fig. 3a and 3b.

1.3.3. The Basolateral Complex (BLA)

Cortical Inputs

The amygdala receives sensory information from the respective cortices of all major sensory modalities. Olfactory and gustatory/visceral information (which are only rarely used in fear conditioning) has access to the amygdala at an earlier stage of cortical processing than visual, auditory and somatosensory information (McDonald, 1998). For the latter, this means that information has already been processed by a series of cortico-cortical projections originating in the primary sensory cortex of each modality before being transmitted to the BLA. To elaborate, a visual stimulus is transmitted to the amygdala via the thalamus (lateral geniculate body or lateral posterior nucleus) →V1,V2 (primary and second visual cortex) →Te2(temporal cortical area2)/perirhinal cortex → LA (Shi and Davis, 2001). Perirhinal and enthorhinal cortices, which maintain strong

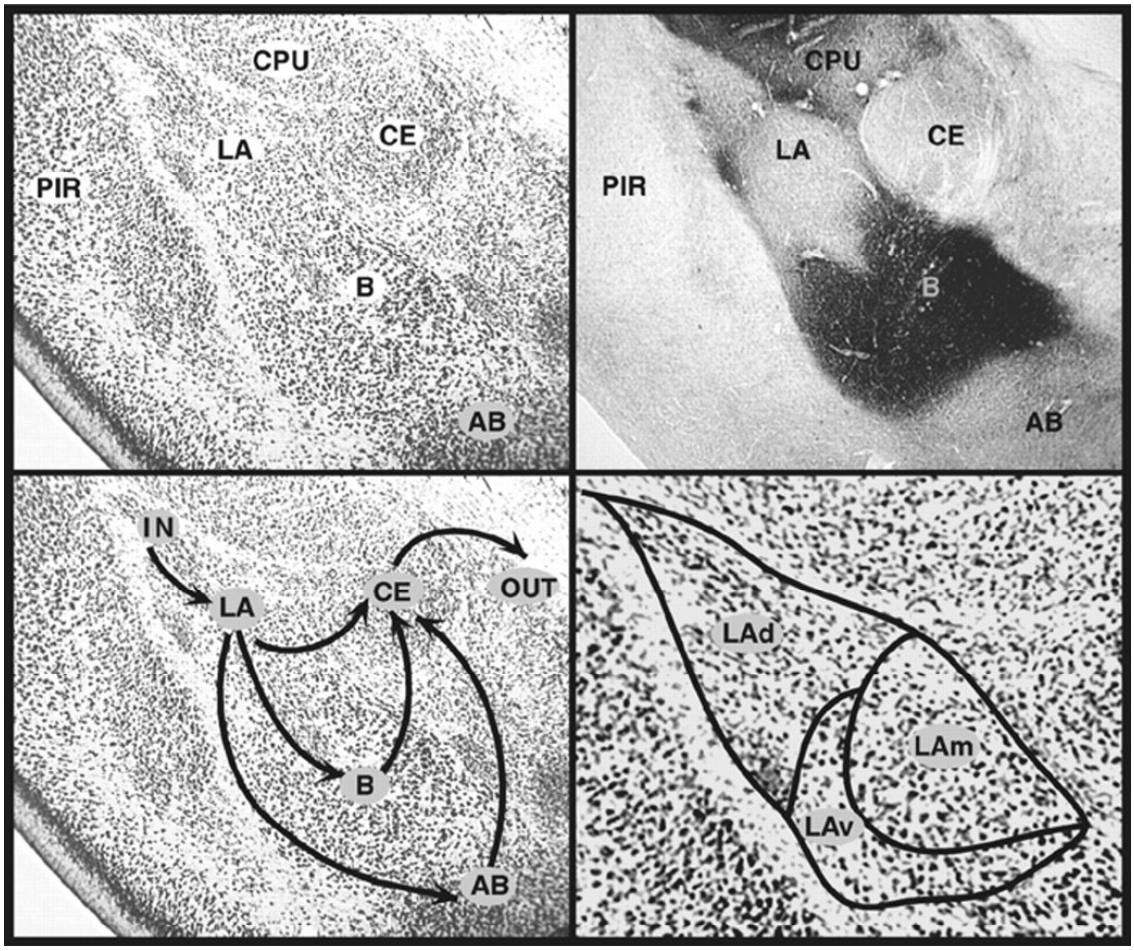


Figure 3a The regions of most relevance to the pathways of fear conditioning are the lateral (LA), basal (B), accessory basal (AB), and central (CE) nuclei. The piriform cortex (PIR) lies lateral to the amygdala, and the caudate-putamen (CPU) is just dorsal to it. Comparison of the Nissl-stained section (*upper left*) and an adjacent section stained for acetylcholinesterase (*upper right*) helps identify the different nuclei. The major pathways connecting LA, B, AB, and CE are shown (*lower left panel*). (*Lower right*) A blowup of the LA, emphasizing the fact that each nucleus can be divided into subnuclei.

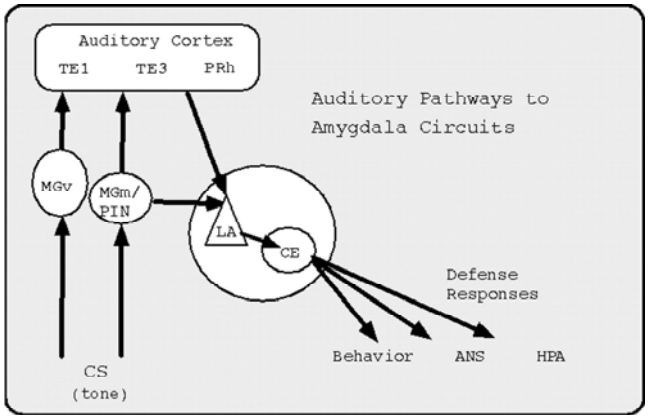


Figure 3b The neural pathways involved in fear conditioning are well characterized. When the CS is an acoustic stimulus, the pathways involve transmission to the lateral nucleus of the lateral amygdala (LA) from auditory processing areas in the thalamus [medial division of the medial geniculate body (MGm/PIN)] and cortex [auditory association cortex (TE3)]. LA, in turn, projects to the CeA, which controls the expression of fear responses by way of projections to brainstem areas. ANS, Autonomic nervous system; CS, conditioned stimulus; HPA, hypothalamic-pituitary axis; MGv, ventral division of the medial geniculate body; PRh, perirhinal cortex; TE1, primary auditory cortex.(LeDoux, 2000).

projections to the BLA, represent polymodal cortical areas (McDonald, 1998). Contrastingly, olfactory inputs arise from the olfactory bulb and primary olfactory cortex (piriform cortex) (Carmichael et al., 1994; McDonald, 1998) and reach the LA without elaborate processing by cortical cascades.

In all species, most of the cortico-amygdala projections enter the subcortical white matter, including the external capsule, and then take the most direct route to the amygdala (Mascagni et al., 1993). Cortico-amygdala projections are glutamatergic as demonstrated by stimulation experiments (Femano et al., 1983) and electron microscopic investigations (Brinley-Reed et al., 1995), in which cortico-amygdala axons were shown to form asymmetric synapses with amygdalar neurons.

Thalamic input

A direct thalamo-amygdala projection has been described for the visual stimulus (Shi and Davis, 2001) coming from the lateral posterior nucleus of the thalamus and terminating in the LA nucleus. CS pathways that involve auditory inputs are shown to arise in the posterior intralaminar nucleus (PIN) of the thalamus and likewise to project directly to the LA nucleus (Doron and LeDoux, 2000).

Cortical vs. thalamic pathway

Since thalamic and cortical CS inputs both converge on the BLA complex, the question arises as to how these two pathways differ. LeDoux proposes (LeDoux, 2000) that the direct thalamo-amygdala path ensures faster transmission, but is unable to benefit from cortical processing – with the result that it can only provide the amygdala with a crude, almost archetypal representation of the stimulus. This “quick and dirty road” allows the brain, however, to start to respond immediately to the possible danger, eliciting for example a reflexive behaviour such as flight. Meanwhile, another part of the thalamus sends (more detailed) sensory information to the respective cortical area, where a more accurate representation of the stimulus is produced and perhaps associated information is gathered. The outcome of this cortical processing is then fed to the amygdala as well (LeDoux, 2000).

US pathways

Information concerning the aversive properties of stimuli (US) has to be transmitted to the LA for CS-US convergence to occur. The US is most often a somatosensory input, as it conveys nociceptive information. Recent behavioural studies show that such

somatosensory US inputs to the amygdala can come from either thalamic or cortical areas (Shi and Davis, 1999), a finding that parallels the conclusions concerning CS inputs. In particular, nociceptive inputs are relayed to the LA and other amygdala nuclei via projections from the parabrachial complex (Bernard et al., 1993), the dysgranular insular cortex (McDonald, 1998), and midline and intralaminar thalamic nuclei and posterior internuclear nucleus (PIN) (Turner and Herkenham, 1991).

While the LA nucleus is essential for conditioning to a tone or light, the B and AB nuclei play key roles in contextual fear conditioning. In addition to expressing fear responses to the actual CS, rats also exhibit a fearful state when returned to the context (e.g. the chamber) in which the conditioning experiment occurred (Frankland et al., 1998; Maren and Fanselow, 1995). Conditioning to a contextual cue involves the representation of the context by the hippocampus, of which the CA1 area and the subiculum maintain projections to B and AB nuclei (Canteras and Swanson, 1992). These contextual representations, relayed from the hippocampus to the AB, may be subject to modification by the US inputs to the AB (Burstein and Potrebic, 1993). As for tone conditioning, B and AB nuclei project to the CeA that controls the expression of fearful responses.

Outputs: There are four main output projections from the BLA complex. Extensive reciprocal connections exist back to the cortex including the frontal cortex, which might be involved in the conscious perception of fear and anxiety. Unidirectional projections comprise those to the caudate putamen, the nucleus accumbens and the CeA (McDonald, 1991). Projections to the two striatal areas might relay motivationally significant information to motor areas necessary for the avoidance of harmful stimuli associated with primary reinforcers (Davis et al., 1994). Projections to the CeA, the major intra-amygdaloid target of the BLA complex, are crucial for autonomic and reflexive responses and will be described next.

1.3.4. The Central Nucleus (CeA)

Lesions of the CeA block the expression of fear conditioned responses, establishing the CeA as the main source of amygdala outputs to brain stem and hypothalamic sites which are capable of influencing or initiating autonomic and somatic components of fear reactions (Davis et al., 1994; Gallagher et al., 1990; LeDoux, 2000). Importantly,

stimulation of the CeA produces the constellation of conditioned fear responses even in the absence of prior fear conditioning (Kapp et al., 1982), indicating that the complex behaviour pattern of fear responses is probably "hard wired". Thus, it is only necessary for the conditioned stimulus to activate the CeA; the CS-US association occurs in or before the CeA

Input pathway: The question, how information received by the LA reaches CeA, has not been answered completely. The LA evokes conditioned fear responses either via transmitting the information to the B and/or AB nucleus and from there to the CeA or via a direct projection from the LA to the CeA (LeDoux, 2000) (see also Fig. 3a). Yet there are unresolved questions concerning both pathways. The CeA is composed of several subnuclei, only one of which contributes significant projections to the brain stem: the medial sector of the CeA (CeM) (Petrovich and Swanson, 1997). The difficulty comes from the fact that the LA has little if any projections to the CeM, but rather projects to the lateral (CeL) or amygdalo-striatal sectors (Krettek and Price, 1978; Pitkanen et al., 1995). To reconcile these findings, it was suggested that the CeL projects to the CeM, but this projection is relatively minor and GABAergic (Pare and Smith, 1993). Given that chemical or electrical excitation of CeM elicits the behavioural correlates of fear, GABAergic input from CeL would decrease rather than augment fear expression. As for the second option, an indirect projection from LA via B to CeA, a substantial amount of evidence exists indicating that there are indeed massive projections from the LA to B which in turn projects to all sectors of the CeA (Krettek and Price, 1978; Pare et al., 1995; Pitkanen et al., 1995). However, pretraining excitotoxic and electrolytic lesions of B do not interfere with the acquisition of conditioned fear (Nader et al 2001). Taking into consideration all these observations, (Pare et al., 2004) proposed a revised model with the paracapsular intercalated cells at its core.

Output pathways: Different sites in the hypothalamus and brain stem are responsible for specific fear reactions as illustrated in Fig. 4. For example, the increase in blood pressure is mediated by the lateral hypothalamus, stress hormones or endocrine responses in general are evoked by the paraventricular hypothalamus while freezing and startle response are mediated by the central gray and nucleus reticulopontis caudalis, which are both parts of the brain stem.

With the basic amygdala pathways laid out, next, the basic cell types in the BLA complex and CeA will be characterized.

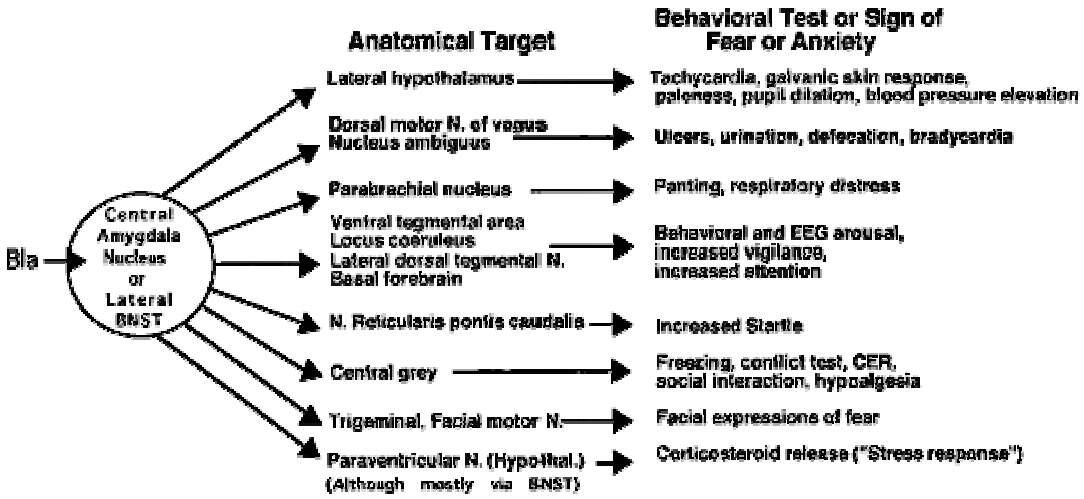


Figure 4 Schematic diagram of the outputs of the CeA to various target structures and possible functions of these connections. (Davis and Whalen, 2001).

1.4. Basolateral Complex: Cell types and their Physiological Properties

As in the neocortex and hippocampus, two main cell types are found in the BLA: pyramidal or projection cells and interneurons.

1.4.1. Projection Cells

This cell type comprises ~85% of all cells in the BLA complex and is the source of most, if not all, of the internuclear projections within the amygdala (Smith and Pare, 1994). They are spiny multipolar, often pyramidal-shaped cells with highly collateralized axons that use glutamate as their transmitter. Unlike their cortical counterparts, these cells are not arranged with parallel apical dendrites but are randomly oriented, particularly close to the nuclear border (Rainnie et al., 1993). Their soma diameters average from 10 – 15 μm in the lateral nucleus and from 15 – 20 μm in the basal nucleus in the rat (McDonald, 1982). Electrophysiologically, projections cells can be identified by their broad action potentials that show varying degrees of spike frequency adaptation in response to prolonged depolarizing current injection (Faber et al., 2001; Kroner et al., 2005). In addition, they display intrinsic voltage-dependent oscillation, demonstrated in intracellular recordings in LA neurons *in vitro* as well as *in vivo* (Pape et al., 1998; Pare et al., 1995).

The excitability of BLA projection neurons is tightly regulated by GABA-mediated IPSPs, composed of a fast component mediated by $GABA_A$ receptors (see chapter 1.8) and a slow component mediated by $GABA_B$ receptors (Rainnie et al., 1991; Washburn and Moises, 1992). Indeed, large numbers of synaptic boutons immunoreactive for GAD are found concentrated strategically around the soma, proximal dendrites and axonal initial segment of projection cells (Carlsen, 1988; Pare and Smith, 1993). The IPSPs are thought to result primarily from the activity of BLA interneurons, as lesions deafferenting the BLA complex lead to minor decreases in GAD levels (Le Gal LaSalle et al., 1978). Moreover, although other amygdaloid nuclei contain GABAergic interneurons, internuclear projections linking the different nuclei of the BLA complex appear to consist only of excitatory projections (Samson et al., 2003; Smith and Pare, 1994).

Because inhibition plays such an essential role in the BLA complex, it will be discussed separately in section 1.5.

1.4.2. Interneurons

Interneurons in the amygdala comprise a heterogenous class of sparsely spiny or aspiny local–circuit neurons that use GABA as a transmitter. Yet while the characterization of cortical and hippocampal interneurons has advanced rapidly by correlating morphological with physiological data, the classification of amygdala neurons has been rather sluggish mainly due to the paramount difficulty of identifying them. In fact, they possess neither a conspicuous shape, (they span the whole spectrum from round to pyramidal-like), nor do they localize in a specific area (though they are more frequent in B than in LA). Thus, the rare data available on amygdala interneurons falls basically into two sets, morphological and physiological, which are only poorly correlated.

Immunohistochemical studies detecting calcium binding proteins and /or neuropeptides provided a first morphological classification since such proteins are typically expressed by interneurons. Double-labelling studies suggest that there are at least four different subpopulations of interneurons:

1) parvalbumin (PV)-positive neurons (most of which also contain calbindin);
2) somatostatin-positive neurons (most of which also contain calbindin and/or neuropeptide Y);
3) large multipolar cholecystokinin (CCK) positive neurons that are often calbindin-positive, and
4) small bipolar and bitufted interneurons that exhibit extensive colocalization of vasoactive intestinal polypeptide, calretinin, and CCK (Mascagni and McDonald, 2003; McDonald and Mascagni, 2001; McDonald and Mascagni, 2002).

Further research has revealed that PV-positive interneurons form a pericellular basket or axonal cartridge around the perikarya and initial segment of projection cells in the amygdala (McDonald and Betette, 2001).

In a study in the rat amygdala using the Golgi impregnation technique, two subtypes of interneurons were identified in the BLA complex by their morphology (McDonald, 1982):

1) chandelier cells with their characteristic clusters of axon varicosities that form synapses with the axon initial segment of pyramidal cells, and

2) neurogliaform cells, remarkably small (~10 μm) cells with a restricted spherical dendritic tree and branching axons that travel little further than the confines of their dendrites.

Relatively little is known about the innervation pattern of amygdala interneurons. A large fraction of interneurons, the PV-positive interneurons, are targeted by only a few cortical synapses yet by numerous excitatory synapses of BLA projections cells (McDonald et al., 2005; Smith et al., 2000), indicating a role for these interneurons in feedback inhibition reminiscent of PV-positive interneurons in the hippocampus. However, interneurons that mediate feedforward inhibition, receiving excitatory thalamic or cortical innervation, have not been as unambiguously identified; in fact up to now only three studies have specifically dealt with this issue: an excitatory thalamic input on amygdala interneurons is demonstrated in a tract tracing study, yet functional data confirming this connection are still lacking (Woodson et al., 2000); *in vivo* stimulation in the enthorhinal cortex elicits activity in cat amygdala interneurons (Lang and Pare, 1998); convergent excitatory inputs on amygdala interneurons from presumed cortical and thalamic sources have been observed in a study using slice preparation and rather crude stimulation conditions (Szinyei et al., 2000). Moreover, all three studies fall short of a further characterization of the interneuron targeted by the excitatory afferents.

Likewise, conflicting data has been gathered concerning the glutamatergic receptor profile on BLA interneurons. (Mahanty and Sah, 1998) have claimed that glutamatergic inputs to interneurons, identified solely by their spiking pattern, are mediated exclusively by AMPA receptors – an unprecedented feature in the forebrain. Furthermore, in contrast to AMPA receptors at inputs to pyramidal neurons, these receptors are calcium permeable due to their lacking of the GluR2 subunit, allowing for a novel NMDA-independent form of LTP (Mahanty and Sah, 1998). This finding has not been reproduced, though; a more recent study found a regular NMDA-mediated response in all BLA interneurons recorded from (Szinyei et al., 2003), thus raising the question if two distinct subtypes of interneurons exist in the BLA, one carrying only AMPA receptors, the other endowed with AMPA and NMDA receptors.

1.5. Inhibition As a Major Determinant of BLA Activity

The fact that the inhibitory circuitry plays an unusually powerful role in this structure is strikingly evidenced by the finding that although the cellular composition of the BLA complex is similar to the cerebral cortex, and both structures are subjected to the same neuromodulatory inputs (Aggleton, 1992), BLA projection neurons exhibit extremely low firing rates with the majority of projection neurons being virtually silent unless presented with a specific sensory stimulus (Gaudreau and Pare, 1996). Physiological studies have stressed the paucity of spontaneous activity (<1 Hz) in projection cells in unanaesthetized animals in contrast to cortical pyramidal cells that fire at ~10 Hz during wakefulness (Pare and Gaudreau, 1996). The silence of projections cells is especially remarkable because the LA is endowed with extremely divergent intrinsic connectivity. The axon collaterals of these cells bear numerous varicosities that form *en passant* asymmetric synaptic contacts, typically with dendritic spines. These varicosities occur every 5-10 μm, suggesting that each projection cell forms 100-200 excitatory synapses per millimetre of axon, most of which end on other projection cells (88%) (Smith and Pare, 1994). Despite this massive excitatory connectivity, *in vitro* and *in vivo* intracellular studies have reported that afferent stimulation elicits an initial excitatory postsynaptic potential (EPSP) that is truncated by a large amplitude hyperpolarizing potential in projection cells, composed of a distinct "early" $GABA_A$ and a "late" $GABA_B$ mediated IPSP (Lang and Pare, 1998; Rainnie et al., 1991). Indeed, increasingly strong stimulation of BLA afferents pathways *in vivo* paradoxically produces greater inhibition of BLA projection neuron (Lang and Pare, 1997) with the result that only a narrow range of low stimulus intensities can trigger spikes in projection cells (Lang and Pare, 1998).

A major factor contributing to the suppression of projection cell activity is the relatively low level of inhibition directed towards interneurons. In contrast to projection cells, interneurons display a depolarized resting potential that is punctuated by numerous spontaneous EPSPs and spikes (spiking frequency ~ 15 Hz) but relatively few IPSPs (Lang and Pare, 1998). In addition, their reversal potential of $GABA_A$-IPSPs is depolarised with respect to that of projection cells. This difference arises from cell-type specific chloride transporters, KCC and NKCC, which extrude chloride in projection cells and accumulate chloride in interneurons respectively (Martina et al., 2001). Furthermore, GABAergic interneurons (at least PV-positive ones) receive a significantly lower

proportion of GABAergic synapses than do projection cells (Smith et al., 1998). For projection cells, both *in vivo* and *in vitro* studies in the LA have demonstrated that a synaptically activated K_{Ca} conductance, which does not occur in interneurons, is a major inhibitory component (Danober and Pape, 1998; Lang and Pare, 1997). This conductance is activated by calcium influx via NMDA receptors and plays an essential role in preventing paroxysmal depolarizations that underlie epileptic seizures (Danober and Pape, 1998).

1.6. Central Nucleus (CeA): Cell Types and their Physiological Properties

The CeL and CeM are conspicuously cell-dense areas that contain mainly medium-sized, spine-dense neurons, many of which use GABA as their neurotransmitter (Pare and Smith, 1993) and express a variety of neuropeptides, ranging from corticotropin-releasing factor (CRF), and enkephalin to somatostatin, substance P and galanin (Sun and Cassell, 1993). On average, cells of the CeM exhibit a lower spine density and longer dendrites than cells of the CeL (McDonald, 1982). All CeA neurons receive remarkably dense GABAergic input that is thought to arise chiefly from the centromedial group and the intercalated cells (Pare and Smith, 1993), since lesions of stria terminalis and BLA complex do not diminish the concentration of GABAergic terminals in the CeA (Sun and Cassell, 1993). This concept is supported by electrophysiological studies showing that stimulation of CeL evokes GABA-mediated IPSPs in CeM neurons (Nose et al., 1991) and stimulation of intercalated cells triggers GABA-mediated IPSPs in CeL and CeM (Royer et al., 1999).

Confounding data exist concerning the glutamatergic or GABAergic nature of output neurons from the CeA. Synaptic contacts between axon terminals arising in the CeA and adrenergic neurons of the ventrolateral medulla are usually of the symmetric (inhibitory) type (Cassell and Gray, 1989). However, some anatomical and electrophysiological studies suggest, that in contrast with medium spiny GABAergic neurons of the striatum, projection cells of the Ce targeting the parabrachial nuclei are not GABA-immunoreactive (Sun and Cassell, 1993). Considering the variety of brainstem and hypothalamic targets of the CeA, these contradictory results could be reconciled by the hypothesis that central

amygdalar efferents are generally capable of releasing either glutamate or GABA depending on the target and/or circumstances.

As for inhibitory receptors in the CeL, two distinct types of ionotropic GABA receptors have been demonstrated in the rat:

1) A typical $GABA_A$ receptor that can be blocked by low concentrations of bicuculline and is positively modulated by benzodiazepines and barbiturates.
2) A bicuculline-resistant GABA receptor that is blocked by the specific $GABA_C$ receptor antagonist TPMPA (Delaney and Sah, 1999) and thus has been called $GABA_C$-like-receptor due to its pharmacological similarity to retinal $GABA_C$ receptors ($GABA_C$ receptors are composed of ρ homomers (Enz et al., 1995)).

Interestingly, the $GABA_C$ receptor is negatively modulated by benzodiazepines such as diazepam (Delaney and Sah, 2001), leading the authors to suggest that an alternative mechanism for the anxiolytic actions of benzodiazepines might exist in the CeA.

1.7. The Intercalated Cells

The intercalated cell groups, or massa intercalata, were so named because they appear to be discrete clusters of cells intercalated (= inserted) among major amygdaloid nuclei. They constitute by far the densest population of strongly GAD- or GABA-positive cell bodies in the entire amygdaloid complex (Pare and Smith, 1993) and have been demonstrated in a variety of mammals such as the rat, guinea pig, mouse, rabbit, opossum, cat, horse, cow, pig, macaque and human (Millhouse, 1986).

The largest intercalated cell group hangs from the BLA nucleus and, remaining ventral to the CeA, reaches across to the medial nucleus. Viewed in a sagittal plane, this cell group extends dorsally over the rostral pole of the basolateral nucleus and rostrally beneath the anterior commissure (Millhouse, 1986). Other intercalated cell groups (later termed paracapsular cells (pcs) by Alheid et al., (1995) occur either as thin sheets or as small cell clusters interposed a) between the BLA complex and the CeA nucleus along the intermediate capsule, b) along the external capsule and c) between the lateral and the basal nucleus (Millhouse, 1986).

All of the intercalated cell groups are made up of the same two cell types: the vast majority of intercalated cells, designated as medium neurons, are GABAergic, small,

spiny neurons with a diameter of 7-12 μm in rats, mice and guinea pig (Millhouse, 1986) or 9-18 μm in the cat (Pare and Smith, 1993), while located among them are a few larger cells (Millhouse, 1986), some of which are cholinergic (Nitecka and Frotscher, 1989). Tract-tracing studies have revealed that the main intra-amygdaloid targets of the intercalated cell masses are the medial and CeA nucleus of the amygdala, whereby the larger, rostrally located group of intercalated cells projects mainly to the medial nucleus, while the smaller, caudally located ones – the paracapsular cells – project to the CeA. In addition, weaker projections are sent to the globus pallidus, the BLA complex and neighbouring intercalated cells (Pare and Smith, 1993).

Paracapsular cells appear to be particularly densely innervated by glutamatergic afferents since tract-tracing studies demonstrate that axons from the medial prefrontal cortex (McDonald et al., 1996; Sesack et al., 1989; Vertes, 2004) course through the intermediate capsule on their way to other structures or amygdala nuclei, possibly synapsing on intercalated neurons. Furthermore, the more medially located paracapsular cells receive substantial glutamatergic input from the BLA complex (Krettek and Price, 1978; Smith and Pare, 1994). That at least some cortical inputs can actually fire intercalated cells was shown by electrical stimulation of the perirhinal area (Collins et al., 1999).

Up to now, functional studies have focussed exclusively on one subgroup of the paracapsular cells, which are located between the BLA complex and the CeA. Based on the anatomical findings, in which the paracapsular cells are targeted by axons from BLA projection neurons (Krettek and Price, 1978; Smith and Pare, 1994) while extending their axons into the CeA, these cell clusters were hypothesized to constitute an inhibitory gate between the main input and output stations (BLA and CeA, respectively) of the amygdala (Pare and Smith, 1993). Electrophysiological studies supported this concept, as electrical stimuli in the BLA nucleus elicit EPSPs in the paracapsular cells, which then inhibit CeL and CeM neurons (Royer et al., 1999). Interestingly, in the guinea pig a lateromedial correspondence exists between the position of these cells, their projection site in the CeA nucleus and the source of their afferents in the BLA complex. Furthermore, paracapsular cells are interconnected in a lateromedial direction (Royer et al., 2000). Consequently, feed-forward inhibition mediated by paracapsular cells can be increased or decreased depending on which combination of amygdala nuclei (lateral, basolateral) is activated; the

output of the CeA would therefore depend not only on the intensity of sensory input but also on its timing and origin (Royer et al., 2000).

Paracapsular cells seem to be specifically endowed to modify the amount of feed-forward inhibition onto CeA cells, as they express an unusual K current (termed I_{sd} for slowly deinactivating) that activates in the subthreshold range of V_m, inactivates in response to suprathreshold depolarizations and very slowly deinactivates upon return to rest (Royer et al., 2000). Thus, after episodes of suprathreshold activity, the paracapsular cells enter a self-sustained state of heightened excitability associated with increased input resistance and membrane depolarization, which in turn increases the likelihood that synaptic activity will trigger action potentials. This feature enables paracapsular cells to "remember" their recent firing history and modify their activity accordingly.

Another striking characteristic of these cells is their extremely scarce somatic GABAergic innervation (Pare and Smith, 1993), demonstrated by electron microscopic observation, which should permit them to display higher spontaneous discharge rates than the notoriously inactive BLA and CeA cells. In fact, electrophysiological recordings in vivo demonstrate that paracapsular cells fire at much higher rates (~13 Hz) than commonly observed in neighbouring nuclei, suggesting that these cell clusters may provide tonic inhibitory input to their projection sites (Collins and Pare, 1999).

1.8. The $GABA_A$ Receptor

Fast inhibitory synaptic transmission is practically exclusively mediated by $GABA_A$ receptors in the amygdala. Because of their central function, the physiological and pharmacological properties of $GABA_A$ receptors will be presented followed by a description of two of the main neuromodulators of the amygdala inhibitory system, namely, endocannabinoids and dopamine.

$GABA_A$ receptors are fast-acting ligand-gated chloride ion channels, which are composed of five subunits forming a quasisymmetric structure around the ion channel analogous to the structure of the nicotinic acetylcholine receptor. Nineteen $GABA_A$ receptor subunits (α (alpha)1-6, β (beta)1-3, γ (gamma)1-3, ρ (rho)1-3, δ (delta), θ (theta), π (pi), ε (epsilon)) have been cloned from the mammalian CNS, with further variations resulting from alternative splicing (for example for the γ2 subunit). The bulk of experimental

evidence suggests that the predominant $GABA_A$ receptor expressed in adult brain has the molecular composition $(\alpha1)_2$ $(\beta2)_2$ $(\gamma2)_1$ followed by $(\alpha2)_2$ $(\beta3)_2$ $(\gamma2)_1$ and $(\alpha3)_2$ $(\beta3)_2$ $(\gamma2)_1$ (Fritschy and Brunig, 2003) with the subunits arranged pseudo-symmetrically around the ion channel in the sequence γ-β-α-β-α. Yet the diversity of subunits could theoretically give rise to over fifty different subtypes of $GABA_A$ receptors with multiple subunit splice variants increasing the number of possible configurations even further.

In the adult brain, the expression pattern of $GABA_A$ receptor subtypes exhibits a remarkable region- and neuron-specificity, suggesting that individual subtypes are present in distinct neuronal circuits. Support for this concept comes from the specificity of diazepam (see *Benzodiazepines*) action in knock-in mice carrying diazepam-insensitive $GABA_A$ receptor subtypes (Rudolph et al., 2001). Such mice were created by exchanging the conserved His101 residue with an Arg residue in the diazepam binding site. Abolition of diazepam binding on α2-$GABA_A$ receptors *in vivo* results in selective suppression of the anxiolytic effect of this drug (Low et al., 2000), indicating that such receptors are strategically located in circuits mediating the anxiolytic action of diazepam. Likewise, another set of diazepam actions comprising anterograde amnesia, sedation and the majority of the antiepileptic effects is abolished in mice carrying α1 H101R GABA receptors, suggesting a role for this subunit in circuits mediating these effects (McKernan et al., 2000; Rudolph et al., 1999).

The specificity of diazepam action might be causally related to the synapse-specific distribution of $GABA_A$ receptors. Indeed, in neurons expressing several $GABA_A$ receptor subtypes such as hippocampal pyramidal neurons these receptors are segregated to specific locations on the cells surface (Nusser et al., 1996). α1-$GABA_A$ receptors are located postsynaptically in a majority of somatodendritic synapses, and to a lesser extent in the axon initial segment. Contrastingly, α2-$GABA_A$ receptors are particularly abundant in the axon initial segment, while they are rare in somatodendritic synapses (Fritschy et al., 1998; Nusser et al., 1996). α5-$GABA_A$ receptors have an extrasynaptic localization, being distributed throughout the somatodendritic compartment without aggregating at postsynaptic sites (Crestani et al., 2002). The segregated distribution of α1- and α2-$GABA_A$ receptors correlates with differential kinetics of deactivation of these receptor subtypes. Indeed, α1-$GABA_A$ receptors are characterized by faster kinetics of

deactivation and/or desensitization than α2-$GABA_A$ receptors (Brussaard et al., 1997; Juttner et al., 2001).

Regarding the receptor-agonist interaction, GABA binding sites are located at the interface between the α and the β subunits and two molecules of GABA must bind for full activation of the native receptor channel (Sakmann et al., 1983). The application of GABA results in the opening of the $GABA_A$ receptor channel in bursts and short openings to multiple conductance levels with the 27 pS level being the predominant one (Macdonald et al., 1989). In general, $GABA_A$ receptors possess a relatively low affinity for GABA (~10-20 μM), and desensitize under prolonged application of the agonist, marked by a rundown of the opening frequency of the channel (Zhu et al., 1998). However, $GABA_A$ receptors mediating tonic inhibition such as the α5-$GABA_A$ receptors in hippocampal pyramdial cells, the α4βδ in dentate gyrus granule cells and the α6βδ in cerebellar granule cells have a higher affinity for GABA and a higher desensitization threshold compared with those responsible for phasic inhibition (Adkins et al., 2001; Stell and Mody, 2002).

1.8.1. Multiple Actions of $GABA_A$ Receptors

$GABA_A$ receptors are permeable to chloride and bicarbonate anions (Bormann et al., 1987). The functional outcome of receptor activation depends on the transmembrane distributions of these two anions and on the membrane potential of the cell. In most mature neurons, the activity of the chloride-extruding potassium-chloride cotransporter KCC2 (Payne et al., 2003) results in a chloride equilibrium potential that is more negative than the resting membrane potential V_m. The equilibrium potential for bicarbonate is more positive than V_m, but bicarbonate is much less permeable then chloride. Therefore, $GABA_A$ receptor activation typically results in the net entry of anions and the classically described hyperpolarizing IPSP. In this case, both the increase in conductance (that causes shunting of excitatory inputs) and the hyperpolarization (that sums with depolarization) contribute to the inhibitory effect of GABA, thereby reducing the probability that an action potential will be initiated.

However, a hyperpolarizing GABA response might not be inhibitory if it triggers hyperpolarizing-activated excitatory conductances to produce rebound spikes (Chavas et

al., 2004). Moreover, the response to GABA itself can be depolarizing. This is true for most immature neurons that lack KCC2 and instead accumulate chloride by way of the sodium- and potassium-coupled co-transporter NKCC1 (Payne et al., 2003); and it is also true for some mature neurons. Although inhibition can still occur owing to the shunting effect of the increase in conductance (Gao et al., 1998), the effect of the IPSP depends on its location and timing in relation to excitatory inputs, and the interplay between the respective conductance and voltage changes (Gulledge and Stuart, 2003; Staley and Mody, 1992). In immature neurons, the depolarization might be sufficient to trigger calcium influx, a phenomenon that is implicated in GABA-mediated modulation of neuronal proliferation, migration and synapse formation (Ben-Ari, 2002).

1.9. Pharmacology of the $GABA_A$ Receptor

Picrotoxin, bicuculline and beta-carbolines reduce $GABA_A$ receptor currents, whereas benzodiazepines enhance them by binding to specific sites on the $GABA_A$ receptor.

The plant convulsant *picrotoxin* (active ingredient: picrotoxinin*)* inhibits $GABA_A$ receptor currents by blocking the channel pore, thus exerting a noncompetitve inhibition that is use-dependent (Olsen, 1981). In contrast, another plant convulsant, *bicuculline*, is a competitive antagonist, binding to the same receptor site as GABA. Thus, when the dose of GABA is increased, the inhibition of bicuculline is reduced whereas that by picrotoxin is unchanged.

Benzodiazepines (BZs) are clinically active agents that exert anxiolytic, sedative, anticonvulsant and muscle relaxant effects by increasing $GABA_A$ receptor currents. BZs do not gate $GABA_A$ receptors channels themselves, but allosterically enhance the affinity of the receptor for GABA and its effect on channel function. This effect is reciprocal as binding of benzodiazepine agonists *in vitro* is allosterically enhanced by agonists at the GABA binding site (Macdonald and Olsen, 1994). Consistently, the binding of an inverse agonist such as Ro15-4513, an anxiogenic and proepileptogenic drug, at the BZ site leads to a reduced affinity of the receptor for GABA.

BZs bind to a position located at the interface between the α and the γ subunit, yet only the α1, α2, α3, and α5 together with the γ2 isoform form receptors sensitive to classical BZ ligands, allowing for an elegant differentiation between α-subunits (Benson et al.,

1998). $GABA_A$-receptors can be further distinguished by their differing affinity to zolpidem (α1> α2 = α3>> α5) and various beta-carbolines (α1>α2= α3) (Mohler et al., 1996; Sieghart, 1995).

In patch clamp recordings of inhibitory synaptic signals the BZ effects are immediately apparent as an increase in decay time constant and (at least for synaptically located receptors) in signal amplitude, resulting in an enhanced chloride charge transfer. Results from fluctuation analysis and single channel recordings suggest that the BZ diazepam induces an enhanced opening frequency without altering channel conductance or mean open time (MacDonald and Olsen, 1994; but see (Eghbali et al., 1997)). Using ultrafast ligand exchange techniques, Lavoie and Twyman (1996) found that diazepam enhances pseudo-synaptic (non-steady state) $GABA_A$-receptor currents primarily by accelerating GABA association to its receptor at the first agonist binding site as alterations for the second binding site would significantly alter the open and burst durations. Furthermore, Mellor and Randall (1997) demonstrated that another BZ, flunitrazepam, induced slowing of deactivation due to a decreased rate in ligand unbinding. In conclusion, under non-steady state conditions as encountered at a synapse, the increased ligand binding rate and the decreased ligand-unbinding rate cause increased current amplitude and a slowed decay time, respectively. A prerequisite for this is an incomplete postsynaptic $GABA_A$ receptor occupancy, as under conditions of receptor saturation only the prolonged decay is visible (Hajos et al., 2000). Furthermore, the BZ effect is temperature-dependent with regard to the amplitude but not to the kinetics of GABAergic synaptic signals. Thus, zolpidem increases mIPSC amplitude at room temperature, but exerts no effect on the amplitude at 35°C, though the receptor occupancy remains the same at room and physiological temperature (Perrais and Ropert, 1999). This can be explained by the fact that at higher temperatures the on-rate is increased to the point that the BZ effect is no longer relevant.

1.10. Modulation of Inhibitory Circuits

1.10.1. Cannabinoids

The cannabinoid receptor 1 (CB1) is a seven transmembrane G-protein-coupled receptor expressed among other structures in the neocortex, hippocampus and the amygdala. In these regions CB1 is mainly (though not exclusively) localized on presynaptic terminals of CCK-positive interneurons, and can suppress GABA release, thereby modulating synaptic inhibitory transmission (Katona et al., 2001; Katona et al., 1999). This process dubbed "depolarization-induced suppression of inhibition" (DSI) involves the following steps: anandamide and 2-arachidonylglycerol, two endogenous ligands of the CB1 receptor, are rapidly synthesized postsynaptically from lipid precursors in response to depolarization and consequent Ca^{2+} influx. Acting by way of retrograde signaling, the endocannabinoids cross the synaptic cleft and bind to the presynaptically located CB1 where they suppress neurotransmitter release by decreasing the local release probability of synaptic vesicles (Wilson and Nicoll, 2002). More detailed investigations in hippocampal interneurons have revealed that upon G protein activation the $G_{\beta\gamma}$ is released and directly blocks N-type-calcium channels while P/Q-type-calcium-channels are unaffected (Wilson and Nicoll, 2001). CB1 activation suppresses CCK release in addition to GABA release (Beinfeld and Connolly, 2001) and as CCK generally antagonizes the neural and behavioral effects of opioids, colocalization of CB1 and CCK might be one reason why cannabinoids and opioids have synergistic effects on the brain (Manzanares et al., 1999). Another peculiarity concerns the postsynaptic site, as synapses formed by CCK-containing basket cells on hippocampal pyramidal cells are selectively enriched in α2-$GABA_A$ subunits (Nyiri et al., 2001).

In the rat amygdala the CB1 receptor is expressed at high levels in the BLA complex but is absent in the CeA and medial nucleus (Katona et al., 2001). In contrast to the hippocampus, CB1 is not restricted to CCK+-interneurons, as low levels of CB1 protein expression have been reported on projection cells in the rat BLA complex (McDonald and Mascagni, 2001). In line with these anatomical findings, electrophysiological studies demonstrate cannabinoid-mediated modulation of excitatory synaptic transmission (Pistis et al., 2004).

A clue to the functional role of the cannabinoid system in the amygdala comes from a recent publication, in which CB1-deficient mice show strongly impaired short-term and long-term extinction in auditory fear conditioning, while memory acquisition and consolidation is unaffected (Marsicano et al., 2002). In this study, presentation of the tone during the extinction trials causes an instantaneous rise in endocannabinoid levels exclusively in the BLA complex, but not in the medial prefrontal cortex, though both areas are thought to have central roles in the extinction of aversive memories (Morgan et al., 1993). Given the fact that principal neurons of the BLA and CeM output neurons are inversely correlated in their activity (Collins and Pare, 1999), the authors speculate that a CB1-mediated decrease in local inhibition network activity leads to a disinhibition of principal neurons and finally to an extinction of the freezing response. However, it is not clear at the present time how to reconcile this hypothesis with findings from other studies implicating GABAergic transmission in fear extinction (Harris and Westbrook, 1998).

1.10.2. Dopamine (DA)

Dopamine, a catecholamine neurotransmitter, modulates various aspects of brain function, from control of movement, feelings of reward and reinforcement to hormone secretion. The majority of brain DA cells are located within the midbrain, in the substantia nigra (SN) and the adjacent ventral tegmental area (VTA), and send nerve fibres throughout the forebrain. Commonly, three neuronal pathways are distinguished: The nigrostriatal pathway comprises the axons of the SN neurons projecting to the striatum, and is essential for control of locomotion. The importance of this pathway is highlighted by the extraordinarily high number of DA receptors in the striatum and the drastic consequences resulting from the degeneration of SN neurons as seen in Parkinson's disease. Originating in the VTA, a second fiber tree stretches to limbic structures (e.g. the nucleus accumbens, the amygdala and septum), constituting the mesolimbic pathway, while a third fiber bundle projects to cortical regions (e.g. frontal and cingulate cortex), forming the mesocortical pathway. Whereas the mesocortical pathway has been implicated in attention and other cognitive processes, the mesolimbic pathway is thought to modulate emotion and motivation. Indeed, the latter is considered the major substrate of reward and reinforcement for both natural reward and addictive drugs (Koob et al., 1998; Robbins

and Everitt, 1996), evidenced by the fact that all drugs of abuse directly or indirectly activate this circuit, even though they primarily act on different neurotransmitter systems. The amygdala, in particular the CeA, has been identified as one of the sites in the brain, where addictive drugs such as cocaine – that block DA uptake and thereby increase dopaminergic tone – act during the rush/euphoria phase as well as during craving (Breiter et al., 1997; Breiter and Rosen, 1999).

Although these data strongly suggest a role for DA and amygdala in positive emotions, there is a burgeoning literature on DA-mediated regulation of amygdala-dependent processes in negative affects as well (Pezze and Feldon, 2004). DA, which is released upon stress in the amygdala (Inglis and Moghaddam, 1999), influences different phases of fear learning (Guarraci et al., 2000; Guarraci et al., 1999), whereas DA receptor antagonists block fear learning (Greba et al., 2001; Greba and Kokkinidis, 2000).

Complexity is added to the matter by the existence of five distinct subtypes of DA receptor, which can be subdivided into D1-like (D1 and D5) and D2-like (D2, D3, D4) receptors based on DNA sequence similarities (Missale et al., 1998). All DA receptors are seven transmembrane G-protein-coupled receptors with D1- and D2-like receptors exerting opposing effects on the same signaling pathways: D1 receptor activation through G_s and G_{olf} positively affects adenylyl cyclase with subsequent activation of protein kinase A (PKA), while D2 receptors, acting through G_i and G_o, either uncouple from adenylyl cyclase or negatively affect this enzyme (Stoof and Kebabian, 1982). D1-like as well as D2-like receptors are expressed in the BLA and CeA (Meador-Woodruff et al., 1991) with a particularly high level of D1 receptor mRNA being found in the paracapsular intercalated cells (Asan, 1998). Interestingly, DA activates non-canonical signal transduction pathways in amygdala neurons (Leonard et al., 2003) that do not involve the cAMP/PKA activation but rather protein kinase Src (Loretan et al., 2004).

In general, DA seems to lead to an increased activation in the BLA complex, presumably by reducing cortical feedforward inhibition as shown by *in vivo* and *in vitro* electrophysiological experiments (Rosenkranz and Grace, 1999; Rosenkranz and Grace, 2001). Based on these findings, it was hypothesized that in situations of enhanced DA levels in the BLA, such as during stress and after amphetamine administration, the medial prefrontal cortex regulation of the BLA complex will be dampened, leading to disinhibition of sensory–driven affective responses (Grace and Rosenkranz, 2002). The D1-like receptors seem to play a pivotal role in this process as the attenuation of

inhibition in the amygdala, triggered by stimulation of medial prefrontal cortex afferents, involves activation of D1-like receptors (Rosenkranz and Grace, 2002). In line with this finding, excitability of BLA projection neuron is enhanced via activation of postsynaptically located D1 receptors (Kroner et al., 2005). Confusingly, the same is also true for interneurons in the BLA, in which DA and D1 receptor activation increased evoked firing (Kroner et al., 2005).

D1 receptor activation by the selective partial D1 agonist SKF 38393 enhances both fear learning and expression (Borowski and Kokkinidis, 1998) whereas systemic or focal application of the D1 receptor antagonist SCH 23390 (Greba and Kokkinidis, 2000; Inoue et al., 2000) block acquisition or both acquisition and expression of fear. D1-receptor antagonists are also effective in blocking cocaine self-administration when the antagonist is administered directly into subregions of the extended amygdala such as the CeA (Caine et al., 1995).

Morphologically, a substantial number of dopaminergic fibers can be found running through the BLA complex and the CeA nucleus (Asan, 1998), forming especially dense pericellular arrays (baskets) around the perikarya of PV+ interneurons of the BLA complex (Brinley-Reed and McDonald, 1999). However, by far the densest dopaminergic fiber plexus enwraps the paracapsular intercalated cells (Asan, 1998), indicating a particularly strong dopaminergic modulation for these neurons.

2 Aim of Study

GABAergic synaptic transmission plays a crucial role in amygdala function. However, little is known about either the connectivity of specific amygdala interneurons or their interaction with the different $GABA_A$ receptor types on their target cells. Thus, an important goal in understanding inhibition in the amygdala is the elucidation of convergence of interneuron- and $GABA_A$ receptor-specificity and its influence on amygdala-mediated behavior.

The studies presented in this thesis deal with both aspects of specific inhibitory signaling: the first part is aimed at a better understanding of the distribution of different $GABA_A$ receptors in the amygdala and their targeting by different interneurons, whereas the second part deals with the connectivity and dopaminergic modulation of a specific interneuronal network that mediates the inhibitory influence of the prefrontal cortex over the amygdala.

2.1. Project 1: Mapping of $GABA_A$ Receptors

$GABA_A$ receptors display extensive structural heterogeneity with the subunit composition determining their physiological properties and pharmacological profiles. Indeed, point mutations in the α1, α2, α3 and α5 subunit render the corresponding receptor insensitive to diazepam (Rudolph et al., 2001), and knock-in mice with such point mutations have revealed that particular types of $GABA_A$ receptors are involved in distinct, behaviourally relevant neuronal circuits (Low et al., 2000). Specifically, anxiolytic effects are mediated by α2-$GABA_A$ receptors, whereas α1-$GABA_A$ receptors mediate sedation, anterograde amnesia and most of the antiepileptic effects of diazepam. In addition, studies in the hippocampus indicate that $GABA_A$ receptors with distinct α-subunits are targeted by specific types of interneuron: CCK-positive basket cells expressing CB1 receptors on their axon terminals form synapses enriched in α2-subunits (Katona et al., 2001; Nyiri et al., 2001) in contrast to PV-containing basket cells, which form synapses predominantly via α1-containing $GABA_A$ receptors (Klausberger et al., 2002).

Taking into consideration that anxiolytic effects by diazepam are solely mediated by α2-$GABA_A$ receptors and that the amygdala plays a crucial role in mediating anxiety, the question arises as to where in particular α2- and α1-$GABA_A$ receptors are located in the BLA complex and the CeA, the two nuclei important in fear learning. The expression pattern of α-subunits in the rat brain has been thoroughly investigated by Fritschy and Mohler (1995). According to their study, the most widespread subunits in rat amygdala are α2 and α3, which are frequently coexpressed within individual nuclei such as the CeA, BLA and intercalated cell masses. Remarkably, the α1-subunit is nearly absent from the CeA and the intercalated cell masses, while the α5-subunit is restricted to the basolateral nucleus. However, functional data as to the impact of the individual subunits on inhibitory transmission and their potentiation under diazepam are still lacking; moreover the distribution of α-subunits in the mouse amygdala has not been studied yet. We therefore aim to map the subunit composition of $GABA_A$ receptors functionally and morphologically with regard to the α-subunit. To investigate a possible link between interneuron and receptor diversity, GABA release from a CB1-carrying interneurons will be selectively suppressed by applying CB1-agonists.

2.2. Project 2: The Paracapsular Intercalated Cells

One problem that has hampered studies of amygdala interneurons has been the difficulty of identifying them due to their inconspicuous morphology and location. This limitation can be overcome by using transgenic mice expressing enhanced green fluorescence protein (EGFP) under the control of the promoter for GAD67, a key enzyme for the synthesis of GABA (Tamamaki et al., 2003). Interneurons in these mice can be easily identified by epifluorescence microscopy.

The GAD-positive intercalated paracapsular cells of the amygdala are remarkable in their formation of dense cell clusters outlining the entire BLA complex. They form two main subgroups: one located along the external capsule that has been largely overlooked by researchers, and a better-characterized one between BLA and CeA. Several studies indicate that the latter forms an inhibitory gate between BLA and CeA presumably under the control of medial prefrontal cortical (mPFC) afferents (McDonald et al., 1996; Sesack

et al., 1989; Vertes, 2004). This prefrontal cortical innervation is of special importance since it substantially shapes amygdala-dependent behavior (Hariri et al., 2000; Hariri et al., 2003) and, in particular, ensures appropriate affective responses. Indeed, a disturbed interaction between PFC and amygdala is thought to underlie the affective symptoms of psychiatric diseases such as depression and schizophrenia (Perlstein et al., 2003; Whalen et al., 2002).

Prefrontal cortical control over the amygdala is subject to dopaminergic modulation. By reducing cortical feedforward inhibition, DA produces disinhibition in the lateral amygdala (Rosenkranz and Grace, 2001). This disinhibition can result from reduced excitatory input or from direct suppression of amygdala interneuron excitability. However, interneurons within the BLA were shown to depolarize under DA, increasing rather than reducing spontaneous inhibitory activity (Kroner et al., 2005; Loretan et al., 2004).

Interestingly, the paracapsular cells are a major target of dopaminergic fibers in the amygdala (Asan, 1998). Moreover, they express the highest levels of dopamine D1 receptors in the amygdala (Fuxe et al., 2003) and a moderate level of D2 receptors (Maltais et al., 2000).

Their prominent dopaminergic innervation along with their location along the fibre bundles carrying cortical afferents is suggestive of a substantial involvement of these cells in mediating cortical feedforward inhibition and its modulation by DA observed in the amygdala. Using GAD-GFP-mice, the connectivity and response to DA will be studied in both major clusters of paracapsular cells.

3 Material and Methods

3.1. Electrophysiology

For project 1 five different homozygous mouse lines were tested: α1(H101R) point-mutated, α2(H101R) point-mutated, α1(H101R)-α2(H101R) double point-mutated and α1(H101R)-α2(H101R)-α3(H126R) triple point-mutated mice (Rudolph et al., 2001). Mice with multiple point mutations were obtained by crossbreeding of the original lines. For the reminder of the text these animals will be referred to as wt, α1, α2, α12, and α123, respectively. For project 2, glutamate decarboxylase 67 (GAD67)-GFP (Δneo) mice were generated as described by (Tamamaki et al., 2003). In brief, a cDNA-encoding EGFP was targeted to the locus encoding GAD67 using homologous recombination to specifically express EGFP in GAD67-positive cells of the transgenic mice, because the expression of EGFP is under the control of the endogenous GAD67 promoter in the transgenic mice. Homologous recombinant ES cells were used to generate chimeric male mice. GAD67-GFP mice were obtained by breeding the chimeric male mice with C57BL/6 female mice. The GAD67-GFP mice, which retain a loxP-flanked neomycin-resistance cassette (PGK-Neo), were crossed with cre transgenic mice (Sakai and Miyazaki, 1997) to delete the PGK-neo sequences. The resultant GAD67-GFP (Δneo) mice lack the PGK-Neo cassette, and the expression of EGFP in GAD67-GFP (Δneo) mouse brain was found to be higher than that of EGFP in GAD67-GFP mouse brain (Tamamaki et al., 2003). We used the heterozygous GAD67-GFP (Δneo) mice and called these transgenic mice GAD-GFP mice for simplicity. Because the knock-out of both GAD67 alleles is lethal at birth (Asada et al., 1997) mice heterozygous for the altered GAD67 allele were used for all experiments in this study.

(Both projects) Animals were anesthetized with isoflurane, and killed by decapitation. Brains of mice of either sex, seventeen to twenty-eight days of age, were rapidly removed and put in ice-cold ACSF containing (in mM): NaCl 125, $NaHCO_3$ 26 $NaHPO_4$ 1.25, KCl 2.5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10, equilibrated with 95% O_2 and 5% CO_2. Coronal slices with a thickness of 300-400 μm were cut on a vibrating microtome (Microslicer DTK-1000, DSK Japan and Microm HM 650V; Germany) at 4°C, placed in 32-34°C warm ACSF for 10-20 minutes and then kept at room temperature. Sometimes for preservation

the following substances were added: ascorbinic acid 40 μM, myo inositol 300 μM, pyruvic acid Na salt 200 μM. Slices from GAD-GFP mice were kept in the dark to avoid bleaching.

For project 1 all experiments were carried out at room temperature (22°-24°C) to observe the effect of diazepam on the amplitude of the GABAergic currents, which is temperature dependent (Perrais and Ropert, 1999). For recording, slices were transferred to a chamber mounted on an upright microscope (BX51WI, Olympus Switzerland) equipped with a 20x lens, a fourfold magnification changer, Nomarski type differential interference contrast and infrared illumination. Images were captured with a CCD camera (PCO Vx 45, Till Photonics Germany). Slices were continuously superfused at 1 ml/min with ACSF, kynurenic acid (2 mM) was present throughout the recordings to block excitatory synaptic transmission. Recording electrodes with tip resistances of 3-5 MΩ were pulled from borosilicate glass (GC150TC, Clark UK) on a horizontal puller (Zeitz Instr. Germany). For the kinetic analysis of evoked and miniature IPSCs electrodes were filled with an internal solution containing (in mM): CsCl 100, HEPES 40, $MgCl_2$ 2, MgATP 2, NaGTP 0.3, EGTA 0.1. For the analysis of diazepam effects on evoked IPSCs, electrodes were filled with internal solution containing (in mM): Kgluconate 130, EGTA 1, HEPES 10, MgATP 5, NaGTP 0.5, NaCl 5. Whole-cell recordings were obtained under visual guidance. Only large, pyramidal shaped cells were patched in the BLA complex, in the CeA cells were not chosen according to shape or size. To determine the membrane time constant and the input resistance of the cells, they were held in current clamp configuration and current pulses of –10 pA amplitude were injected for a duration of 1 s. For the kinetic analysis of evoked IPSCs, cells were held in voltage clamp mode at -60 mV and monosynaptic inward-current IPSCs were evoked by 0.3 ms constant voltage stimulation (Grass SD9, AstroMed Inc. USA) through a bipolar stimulus electrode custom made from PTFE insulated platinum-iridium wire with 50 μm diameter (Advent, UK). For iontophoresis, borosilicate glass electrodes were pulled to resistances above 20 MΩ from the abovementioned glass, filled with 3 mM GABA and connected to a constant-current microiontophoresis unit (WPI, USA). Current pulses of 20 ms duration of 500-1000 nA amplitude were used to eject GABA in the vicinity of the soma of the patched cell. To record miniature IPSCs, TTX (1 μM) was added to the perfusate and spontaneous events were recorded continuously for 10-20 minutes. Miniature synaptic currents were detected offline with the 'Mini Analysis' software (Synaptosoft, USA). For the analysis of

diazepam effects on IPSCs, cells were held at –40 mV and outward-current IPSCs were evoked as described above. Signals were filtered at 10 kHz and digitized at 20 kHz (PCI 6035, National Instruments USA) and analyzed on a PC compatible microcomputer with commercially available software (IGOR Pro, WaveMetrics USA). Statistical significance was tested using one-way ANOVA, unless otherwise noted; in case of significance, post-hoc pair wise comparisons were done using Bonferroni statistics (SPSS, Lead technology USA). Data are presented as mean ± standard error of the mean.

For project 2 a second videoimaging camera (VX55 Till Photonics, Germany) and a standard 100 W tungsten lamp connected to an epifluorescence system were additionally installed to visualize EGFP-expressing interneurons in the acute slices. Patch pipettes were pulled from the same glass as abovementioned with tip resistances of 4-8 MΩ. The K gluconate and CsCl intracellular solutions were the same as in project 1. For paired recordings the presynaptic cell was recorded with K gluconate filled patch pipettes and held in current clamp, while the postsynaptic cell was recorded with CsCl containing patch pipettes and held in voltage clamp at –60 mV. Spikes in the presynaptic cell were evoked by current injection of up to 2 nA. For extracellular stimulation glass electrodes with tip resistances of maximal 1 MΩ were pulled from borosilicate glass and filled with 2M NaCl solution. For some experiments 1mM GDPβS was included in the K gluconate internal solution and for reconstruction of the cell, 2 mg/ml biocytin were added to either internal solution. The recording temperature was 30-33°C. All membrane potentials have been corrected for junction potentials of 11 mV for recordings with the K gluconate solution. Recordings with initial resting potentials more positive than –55 mV for interneurons and –65 mV for projection cells were discarded.

Data were recorded with a Multiclamp 700 Amplifier (Axon instruments, USA), filtered at 10 kHz , digitized at 20 kHz (A/D hardware from National Instruments; USA) and analyzed with IGOR Pro software. Statistical significance was tested with two-tailed Student's tests. Series resistance was monitored throughout all experiments by applying a hyperpolarizing pulse every 10 seconds, and if it changed by more than 20% the data were not included in the analysis.

3.2. Immunohistochemistry

Project 1: The distribution of $GABA_A$ receptor subunits in the amygdala was visualized by immunoperoxidase staining of transverse sections from perfusion-fixed tissue in 3-week-old wild type, α1, α2 and α12 mice. Mice were deeply anesthetized with Nembutal (70 mg/kg; i.p.) and perfused through the ascending aorta with 4% paraformaldehyde in 0.15 M phosphate buffer (pH 7.4). The brains were removed, postfixed for 3 hours and incubated overnight in sodium citrate buffer (pH 4.5). They were then irradiated in a microwave oven (650 W; 90 s), cryoprotected with sucrose, and sectioned from frozen blocks with a sliding microtome. Sections were processed free-floating for immunohistochemistry. Incubation in primary antibodies (guinea pig anti-α1, α2- or α3-subunit) was performed overnight as described (Fritschy et al., 1998). Biotinylated secondary antibodies (1:300 Jackson Immunoreasearch, West Grove, PA) were applied for 30 min, followed by Vectastain Elite kit processing (Vector Laboratories, Burlingame, CA) and incubation with diaminobenzidine as chromogen. Sections were mounted onto gelatin-coated glass slides, air-dried, dehydrated, and coverslipped with Eukitt.

Project 2: Immunoperoxidase staining for EGFP was performed in perfusion-fixed tissue from 3-4 week-old transgenic mice, using a polyclonal antibody against GFP (1:80'000; Synaptic Systems, Göttingen, Germany). No specific staining was observed in tissue from wild-type mice. For illustration, digital photomicrographs were taken with a Zeiss Axiocam camera.

Double-immunofluorescence staining was performed with antibodies against GFP, against classical markers of interneurons in the amygdala (parvalbumin, calbindin, NPY, somatostatin, CCK), and against tyrosine hydroxylase (1:10'000; Diasorin, Stillwater, MN), using secondary antibodies coupled to Alexa 488 and Cy3. Images from both markers were acquired sequentially by multitracking with a Zeiss LSM 510 Meta confocal laser scanning microscope, and then digitally merged. For illustration, stacks of 8-12 confocal sections spaced by 0.5 μm were generated using a maximal-intensity projection algorithm.

Co-localization of interneuron markers and GFP was assessed in single confocal sections. Analysis of the distribution of TH-positive axons in the parietal cortex and hippocampus of both wildtype and transgenic mice revealed that presumptive noradrenergic axons were labeled only weakly at the antibody concentration used, whereas presumptive

dopaminergic axons in the cingulate cortex, basal ganglia, and hypothalamus were strongly labeled. Therefore, TH-positive axons in the amygdala likely represent dopaminergic projections.

The distribution of the cannabinoid receptor 1 (CB1) in the BLA complex was visualized by immunofluorescence staining of transverse sections from perfusion-fixed tissue in 4 week-old GAD-GFP-wildtype mice. Mice were anaesthetized with Nembutal (70mg/kg; i.p.) and perfused through the ascending aorta with 2% paraformaldehyde in 0.15 phosphate buffer (pH 7.4) and 15% saturated picric acid. The brains were removed, postfixed for 30 minutes and incubated overnight in 10% DMSO in PBS at 4°C. Sections of 50 μm thickness were cut on a sliding microtome (-40°C) the following morning, mounted on gelatine-coated glass slides and air-dried. Primary antibodies (guinea pig anti-alpha1 (1:8000); anti-alpha-2 (1:700 affinity-purified); rabbit anti-CB1 (1:10000); mouse anti-gephyrin 1:400) were diluted in Tris-Triton (pH 7.4) containing 2% normal goat serum and 2% Triton and applied to the mounted sections (~300 ul) and incubated at 4°C overnight. Slices were then washed three times 15 min in Tris-Triton, before they were incubated in the second antibody (goat anti guinea pig Cy3 (1:500); goat anti rabbit Alexa 488 (1:1000); goat anti mouse Cy5 (1:300)) for 45 minutes at room temperature. Slices were washed again three times 15 minutes in Tris-Triton and then coverslipped with Daco mounting medium.

3.2.1. Processing and Visualization of Biocyin-Filled Cells

Slices were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight. This buffer was used for the whole freezing and visualization procedure and will be referred to as PB from now on. Slices were washed in PB three times 30 minutes and were then incubated in 30% sucrose in PB overnight. They were frozen at –40°C in isopentane and stored at –20°C. For visualization, slices were washed again three times in PB and afterwards incubated for 20 minutes in 1% hydrogen peroxide in PB to inactivate endogenous peroxidase. They were then incubated in permeabilization solution, containing 0.3% Triton and 2% heat-inactivated horse serum in PB, for one hour at room temperature and at 4°C overnight. After washing once in PB, slices were treated with Vectastain Elite Kit (solution A and B were each diluted 1:100 in PB) for four to six

hours. After washing four times 30 minutes in PB and five times 10 minutes in 0.05 M Tris buffer (TB) (pH 7.4), slices were pre-incubated for 15 minutes in 0.05% DAB in 0.05M TB in the dark and cells were finally revealed with 0.003% hydrogen peroxide in 0.05% DAB. When filled cells were visible and/or slices turned brownish, the reaction was stopped by washing twice in ice-cold PB. Slices were mounted on gelatin-coated glass slides and covered with Daco mounting medium (DacoCytomation, USA).

3.3. Drugs

First project: TTX stock solution (Alomone Labs, Israel) was dissolved in citrate buffer at 1 mM. Bicuculline methochloride was dissolved in distilled water to obtain a stock solution at 10 mM. Stock solution of WIN 55,212-2 was dissolved in DMSO at 50 mM. Diazepam stock solution was prepared at 10 mM in DMSO and bath applied. In order to maximize wash-in kinetics we applied diazepam at a concentration of 1 μM, which had been shown to saturate the binding site in susceptible receptors while not interacting with the point mutated receptor. Some experiments were performed with 10 μM diazepam, which caused a slight depression of the inhibitory signal after prolonged application, for brief applications the results were identical with the data obtained using 1 μM and the data were pooled. Diazepam was provided by Hoffmann-La Roche Ltd. (Switzerland), all other chemicals were purchased at Sigma/Fluka (Switzerland), or Tocris Inc. (ANAWA Inc. Switzerland).

Second project: Dopamine hydrochloride was dissolved in distilled water at 70 mM and bath-applied in darkness as it is easily oxidized. Stock solutions of quinpirole (10 mM), SCH 23390 (5 mM), and dihydrexidine (10 mM) were all prepared by dissolving the salt in distilled water. Although it was stored at –20°C, dihydrexidine turned out not to be stable when dissolved, being less effective over time. Thus for this drug, only small numbers of aliquots were made and then immediately used. All chemicals were purchased at Sigma/Fluka (Switzerland), or Tocris Inc. (ANAWA Inc. Switzerland).

4 Project 1: Functional Mapping of $GABA_A$ Receptor Subtypes in the Amygdala

4.1. Results

Evoked inhibitory currents are exclusively mediated by $GABA_A$ receptors

Data were obtained from large, pyramidal-shaped principal cells in the lateral/basolateral amygdala (BLA) and from cells in the central amygdala (CeA) not specifically selected for their morphology. Stimulus electrodes were either placed at the lateral border of, or within the respective nuclei and evoked outward currents were measured at a holding potential of -40 mV (Fig 1A). We tested five different homozygous mouse lines: wt, α1(HR), α2(HR), α12(HR) and α123(HR). After establishing a stable baseline, diazepam (1 μM) was added to the perfusion solution and IPSCs were measured until a stable effect was observed (Figures 1B,C).
The $GABA_A$ receptor antagonist bicuculline methochloride (10 μM) was added to the perfusate to establish the nature of the currents. Bicuculline methochloride blocked the evoked currents in all of these experiments both in the lateral/basolateral amygdala (n=4) and in the central amygdala (n=4) (Figure 2A). In addition, we iontophoretically applied GABA in the central amygdala and measured whole cell currents at a holding potential of -40 mV. Increasing doses of bicuculline methochloride were then added to the perfusate (Figure 2B). The resulting dose response curve gave an IC_{50} of 0.44 μM. At 10 μM, bicuculline blocked iontophoretically evoked currents by 96 ± 1 % (n=5).

Contribution of different $GABA_A$ receptor subtypes to inhibitory currents

We assessed the contribution of the various $GABA_A$ receptor subtypes to the inhibitory signals generated in the target cell, by measuring the effect the different

point mutations had on the diazepam potentiation of the IPSC amplitude (Figure $3A_2$&B_2, Table 1). There was a significant difference between genotypes for the diazepam effect on peak IPSC amplitude (Table 1) ($p<0.01$, one-way ANOVA) in the lateral/basoleral amygdala. The average inhibitory signal in this part of the amygdala was carried by α2- and by α1-subunit containing $GABA_A$ receptors, and maybe a small number of α3-subunit containing $GABA_A$ receptors (Figure $3A_2$). In the central amygdala, (Figure $3B_2$), we again found a significant difference in the response to diazepam between the different strains of mice (Table 1) ($p<0.01$, one-way ANOVA), with almost all of the loss due to the point mutation in the α2-subunit. We were unable to resolve a significant contribution of either α1- or α3-subunit containing $GABA_A$ receptors to the diazepam effect in the central amygdala. Diazepam did not result in a significant increase of the IPSC amplitude in α2(HR) mice (Table 1) and there was no significant difference in the diazepam effect between wt and α1(HR) mice and α12(HR) and α123(HR) mice. Diazepam application also led to an increase in the IPSC decay time constant (Figure $1C_3$ & $2C_3$). The effect of the different point mutations on the diazepam-induced slowing of the IPSC decay was not conclusive (data no shown). No individual point mutation had a significant effect on the increase in decay time constant. Due to the presence of wild type α5-subunit containing $GABA_A$ receptors there was also a residual increase in decay time constant in α123(HR) triple point mutated mice (Figures 1C). Since these receptors are most likely extrasynaptic they did not contribute to the increase in peak IPSC amplitude.

Site	Genotype	Diazepam effect	N	p	Significant difference from
BLA	wt	1.78±0.05	12	<0.01	α1, α2, α12, α123
	α1 (HR)	1.56±0.05	11	<0.01	wt, α12, α123
	α2 (HR)	1.43±0.04	13	<0.01	wt, α12, α123
	α12 (HR)	1.08±0.05	9	0.22	wt, α1, α2
	α123 (HR)	0.96±0.05	7	0.16	wt, α1, α2
CeA	wt	1.40±0.05	11	<0.01	α2, α12 and α123
	α1 (HR)	1.38±0.04	10	<0.01	α2, α12 and α123
	α2 (HR)	1.12±0.06	9	0.06	wt, α1
	α12 (HR)	1.05±0.03	7	0.12	wt, α1
	α123 (HR)	0.99±0.04	8	0.94	wt, α1

Table 1 Summary of the effect of diazepam on peak IPSC amplitude in the BLA and CeA in the different genotype of the mice.

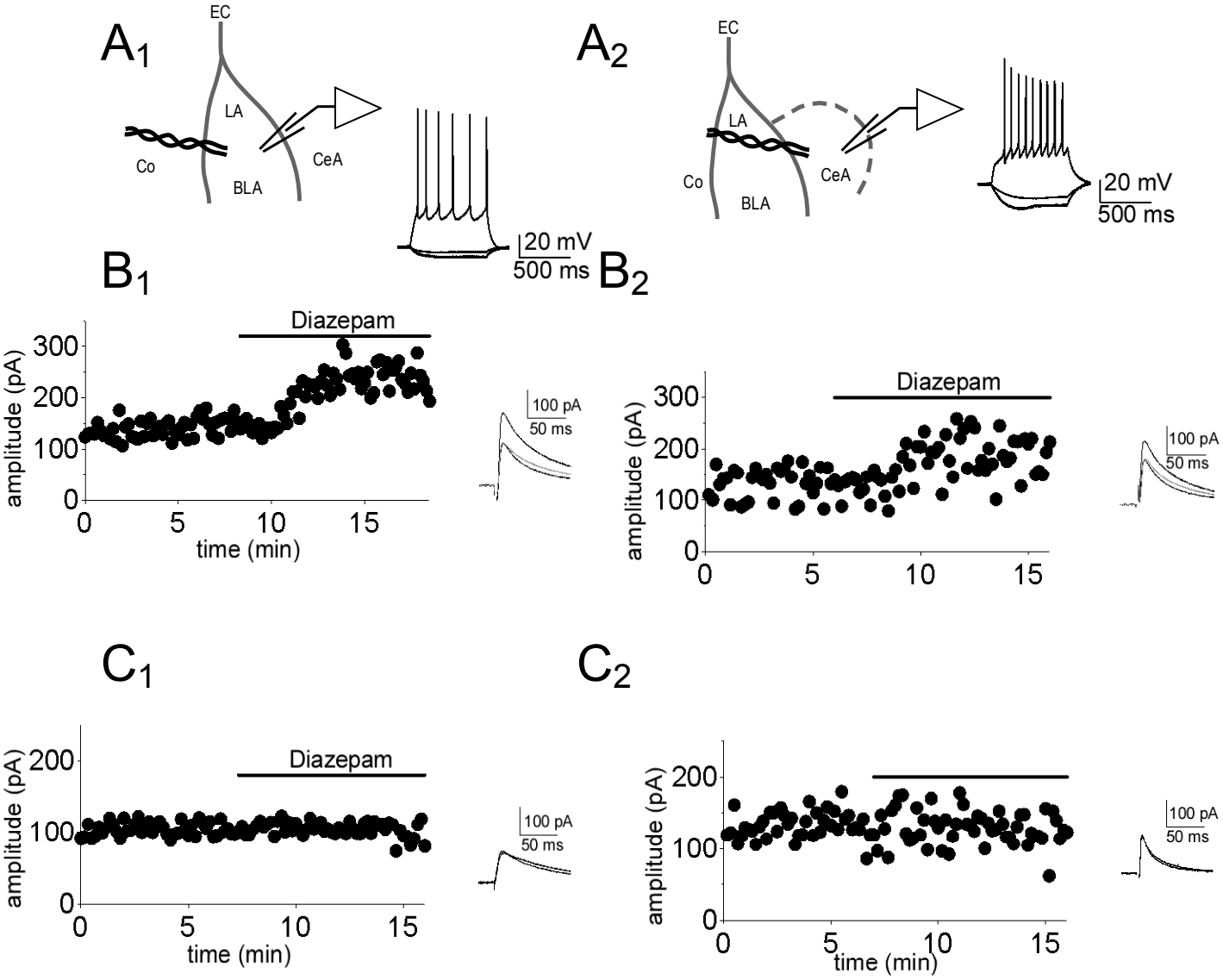


Figure 1 Pharmacology of IPSCs in the LA (left column) and CeA (right column) in wild-type (wt) electrodes. EC external capsule; Co cortex; LA lateral nucleus; BLA basolateral nucleus; CeA central amygdala. (B1) Effect of diazepam (1 μM) in wt mice in the BLA. Plot of IPSC amplitude vs. time, insets show averages of 10-20 traces before and after diazepam. The grey trace represents the scaled version of the diazepam trace demonstrating the decrease in the decay time. (B2) Same data for the CeA. (C1) Lack of effect of diazepam (1 μM) in α1,2,3 triple mutants in the BLA with the same traces as in (B). (C2) The same data for the CeA.

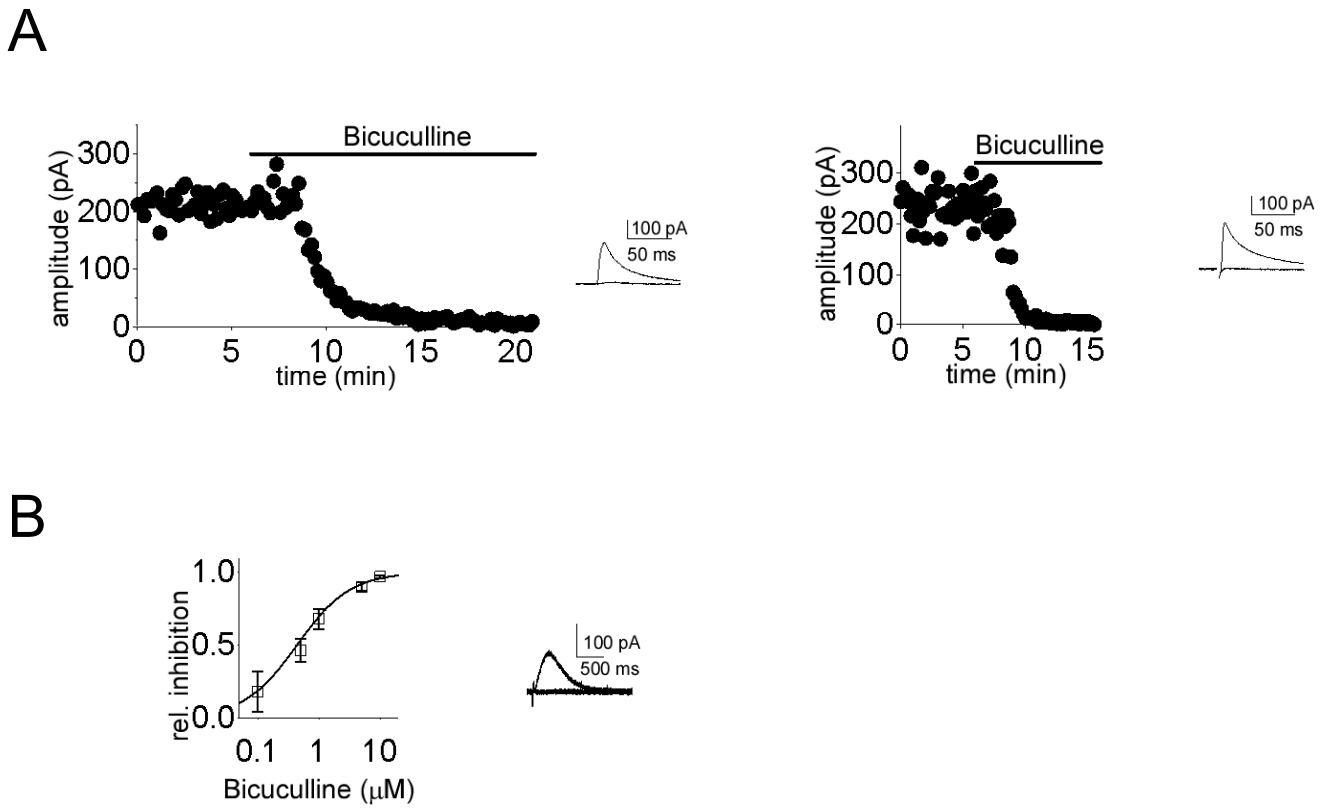


Figure 2 Application of bicuculline methochloride (10 μM) completely blocked the evoked IPSC in LA (left) and CeA (right). (A) Plot of IPSC amplitude vs. time; inset average traces before and during the application of bicuculline. (B) Bicuculline dose-response curve on iontophoretically evoked current (n = 3-6 for each dose) by bicuculline. The resulting curve was fitted (solid line) with $1/(1 + EC_{50}/[\text{bicuculline}])$ with an EC_{50} value of 0.44 μM.

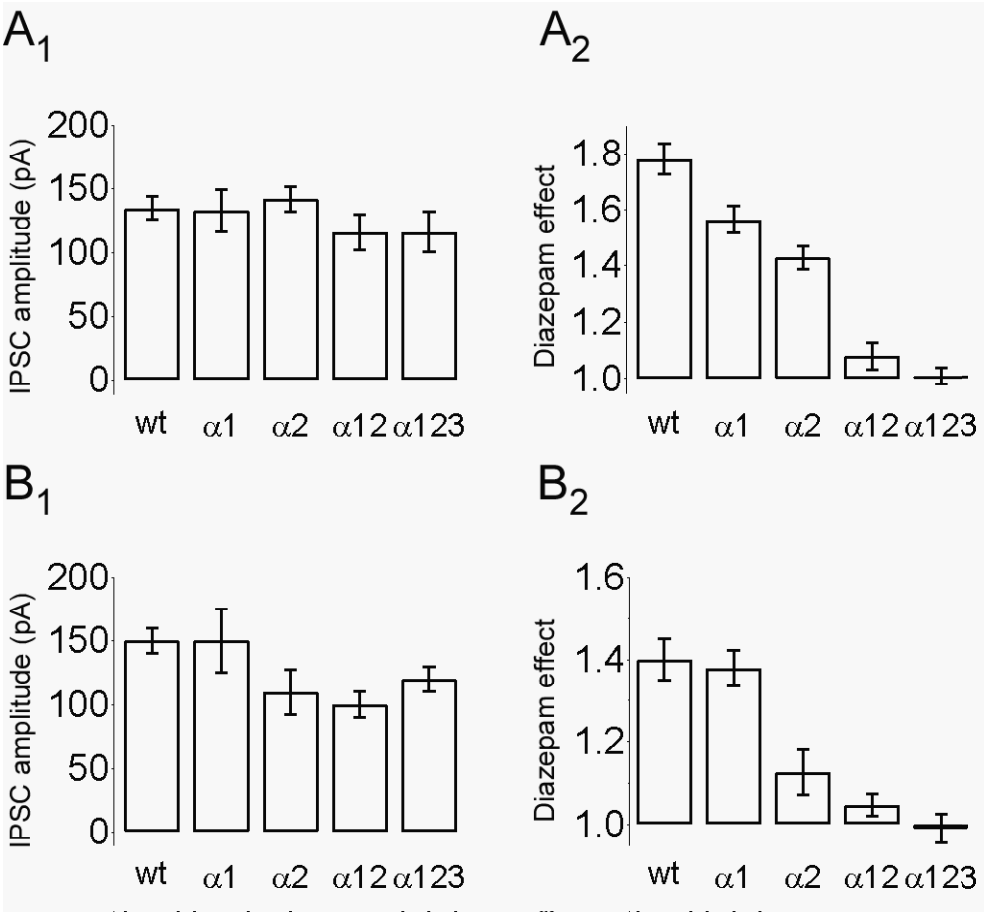


Figure 3 Diazepam effect on IPSCs in the different genotypes of mice. Summary of the results for the study of exracellularly evoked monosynaptic IPSC in the BLA (A) and CeA (B) in five different genotpyes tested: wt; α1 point-muted mice, α2 point-mutated mice, α 1,2 double point-mutated mice, and α 1,2,3 triple point-mutated mice. (A_1 and B_1) The average IPSC amplitude before the application of diazepam. (A_2 and B_2) The relative increase in IPSC amplitude caused by diazepam in the different genotypes.

Immunohistochemical identification of different $GABA_A$ receptor α subunits

To evaluate the $GABA_A$ receptor distribution morphologically, sections from wild type and α12(HR) mice were stained with antibodies against the α1- α2- and α3-subunit of the $GABA_A$ receptor (Figure 4). Due to the different affinities of the antibodies, staining intensities between different subunits cannot be interpreted quantitatively. We noticed a clear difference in the distribution patterns of these α-subunits between the lateral/basolateral and central amygdala. There was no difference in the staining pattern for the α1-subunit (Figure 4) and the α2-subunit (data not shown) observed in wild type and α12(HR) mice, indicating that the point mutations did not influence the distribution of these α-subunits. The most striking finding was the nearly complete absence of α1-subunit immunoreactivity from the central amygdala. Immunoreactivity for the α2-subunit was greater in the central amygdala compared to the lateral/basolateral parts. The α3-subunit was expressed most prominently in the basal amygdala and less in the dorsal nuclei of the lateral amygdala and the central amygdala. In order to detect developmental changes in these distribution patterns we also performed the same staining procedure in adult wild-type mice. There was no difference in the staining patterns observed in three weeks and two months old mice (data not shown).

Specific interneurons signal predominantly through α1-$GABA_A$ receptors

So far we had aimed at stimulating random samples of interneurons in order to determine the contribution of different postsynaptic neurons to receptor diversity. In a second series of experiments we studied select inhibitory terminals onto the same population of neurons in the BLA complex to investigate the role of different presynaptic interneurons in receptor diversity. It is difficult to selectively activate interneurons in the amygdala, since they are not conspicuous; it is, however, possible to depress synaptic transmission from select subgroups of interneurons. Various modulatory G-protein coupled receptors can be found on inhibitory synaptic terminals, and their presence is sometimes characteristic for certain types of interneurons (Poncer et al., 2000). We focused on the type 1 cannabinoid receptor (CB1), since it is clearly expressed only on a subset of interneurons in the lateral/basolateral amygdala (Katona et al., 2001; Marsicano and Lutz, 1999). CB1 receptor knockout mice are deficient in

the extinction of aversive memories (Marsicano et al., 2002), a phenomenon likely mediated by amygdala circuits. Since most of the inhibitory signals in the lateral amygdala are carried by either α1- or α2-subunit containing $GABA_A$ receptors we studied the contribution of $GABA_A$ receptors containing those subunits. Application of the CB1 receptor agonist WIN 55,212-2 (WIN) (5 μM) (Figure 5A) caused a decrease in IPSC amplitude of 34±4% (wt, n=9), 31±7% (α1(HR), n=9) and 35±7% (α2(HR), n=9), respectively, due to the inhibition of GABA release from CB1 positive terminals. Subsequent application of diazepam (1 μM) caused a relative increase in the IPSC amplitude of 1.7±0.04 (wt, n=9), 1.6±0.05 (α1(HR), n=9) and 1.3±0.03 (α2(HR), n=9) (Figure 5A). There was a significant difference between genotypes ($p<0.01$ ANOVA), with a smaller increase in IPSC amplitude in α2(HR) mice ($p<0.01$ post-hoc Bonferroni) compared to both wild type and α1(HR) mice, which were not different from each other (Figure 5B).

Compared to the experiments in which diazepam alone was added (Table 1), application of WIN before the application of diazepam resulted in a more pronounced effect of the α2-subunit point mutation, with a diazepam-induced IPSC amplitude increase of 1.43±0.04 (no pretreatment, n=13) compared to 1.3±0.03 (WIN, n=9). For the wild type and α1(HR) mice there was no difference in diazepam effect between baseline and WIN (Figure 5B). Application of WIN therefore caused a higher proportion of the remaining IPSC to be mediated by α2-subunit containing $GABA_A$ receptors, indicating that there was a predominant reduction in the contribution of α1-subunit containing receptors accompanying the from CB1 containing terminals.

The point-mutation does not affect basic channel functions

To determine whether the point mutation in the α-subunits affected the response of the receptor to synaptically released GABA, we compared mIPSC kinetics and amplitudes recorded within a given region for the different genotypes.

We collected miniature IPSCs (mIPSCs) in the BLA and CeA in three different homozygous mouse lines: wt, α1 point-mutated mice, and α2 point-mutated mice. We used a CsCl-based internal solution and measured mIPSCs as inward currents at a holding potential of -60 mV. There was a trend towards faster mIPSCs in the BLA complex compared to the CeA in all genotypes tested that did not reach statistical

significance, with no difference in mIPSC amplitude between the two regions (Table 2). There was no difference in mIPSC mean amplitude, rise-time or decay time constant between the different genotypes both in the BLA and the CeA (Table 2). Evoked IPSCs recorded under the same conditions had slower rise times and decay time constants than the mIPSCs from the same region and in the same genotype. When comparing the kinetics of evoked IPSCs for each region, we again found no significant differences between the three genotypes of mice tested (Table2).

Using a potassium gluconate-based internal solution, cells patched within the BLA complex had significantly lower input resistances than those in the CeA (353±34 MΩ, n=14 versus 886±190 MΩ, n=7, $p<0.05$ two tailed t-test). There was no significant difference in either membrane time constants (70±5 ms, n=14 versus 80±16 ms, n=7), or the resting membrane potentials (-62.4±1.1 mV, n=14 versus -60.5±1.8 mV, n=7) between neurons from these two regions

Table 2 The properties of miniature and evoked IPSCs in the lateral/basolateral (BLA) and the central amygdala (CeA) in three different homozygous mouse lines.

		Miniature IPSCs				Evoked IPSCs			
Site	Genotype	Amp (pA)	rise time (ms)	τ (ms)	N	Amp (pA)	rise time (ms)	τ (ms)	N
BLA	wt	30.8±0.9	0.86±0.07	20.3±1.1	6	99±14	1.99±0.49	44.8±6.2	5
	α1 (HR)	27.7±2.4	0.77±0.10	21.7±3.4	5	107±8	1.8±0.59	41.7±6.6	4
	α2 (HR)	28.7±3.1	0.85±0.10	21.3±1.6	6	130±43	1.9±0.35	44.2±6.4	5
CeA	wt	28.0±2.6	1.24±0.16	26.4±2.4	5	110±26	1.8±0.36	31.4±3.1	5
	α1 (HR)	29.3±3.7	1.08±0.11	26.3±1.5	4	146±46	2.1±0.33	38.0±4.6	4
	α2 (HR)	30.7±3.8	1.25±0.13	32.3±2.4	5	161±29	2.0±0.28	46.0±7.5	5

Peak amplitudes (Amp), 20%-80% rise times and decay time constants (τ) of miniature and evoked IPSCs were measured in wild type mice and in two different point mutated mouse lines. N indicates the number of experiments performed

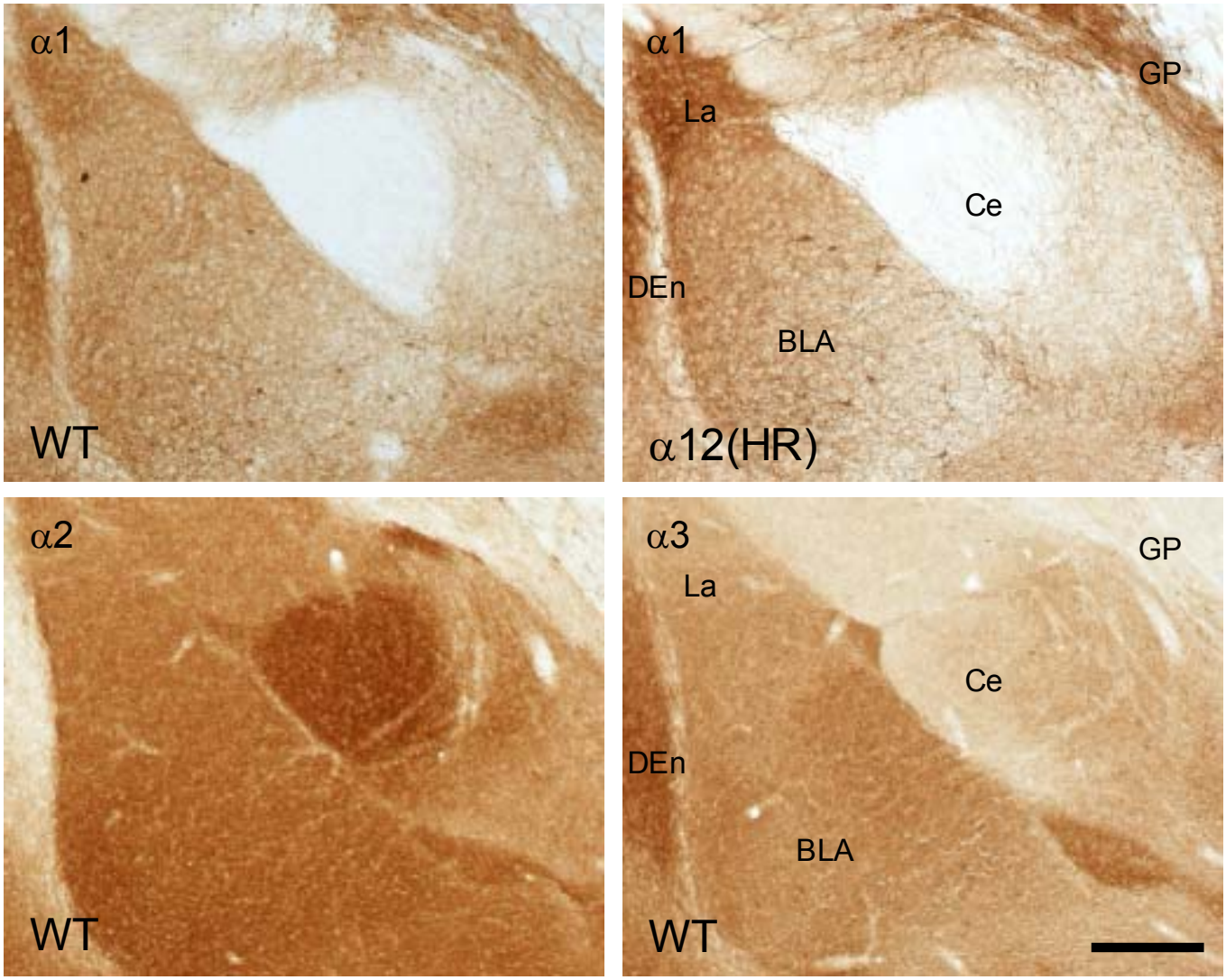


Figure 4 Comparison of the distribution of the α1-, α2- and α3-subunit in the amygdala of 3-week-old wild type mice, as seen in adjacent transverse sections processed for immunoperoxidase staining. The α1- and α2-subunit have strikingly distinct regional and cellular expression patterns. In particular, no α1-subunit-IR is detectable in the CeA and in the intercalated cell masses (I), where the α2-subunit staining is most prominent. Both subunits produce a diffuse labeling of the neuropil in the BLA complex. The α3-subunit is detected in the basolateral nucleus and to a lesser extent in the lateral (La) and central (Ce) nuclei. In addition, the α1-subunit-IR selectively labels a population of neurons, best seen in the ventromedial and lateral parts of the basolateral nucleus. Abbreviations: DEn, dorsal endopiriform nucleus; GP, globus pallidus. Scale bar, 1 mm.

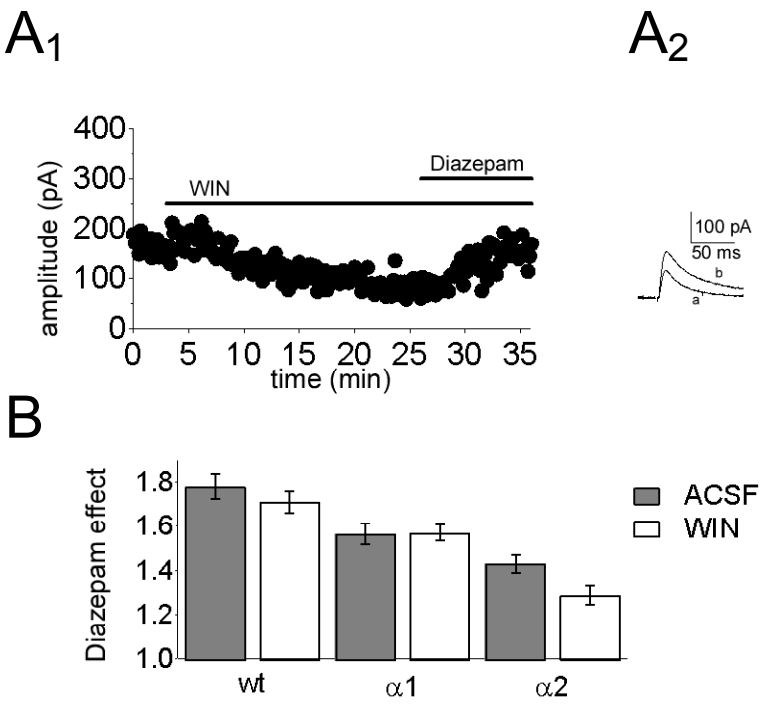


Figure 5 Depression of release from specific terminals in the BLA complex and diazepam application in three different genotypes. (A) Sequential application of WIN 55-212,22 (5 μM) and diazepam (1 μM). (A_1) Plot of IPSC amplitude versus time. (A_2) Average traces during the presence of WIN alone (a) and during the presence of WIN and diazepam (b). (B) Summary of the relative effect of diazepam on IPSC amplitude in the different genotypes. Comparison data (ACSF) are the same as in Figure 3. The only significant difference is an increase in the effect of the α2 point mutation, which results in a larger reduction of the diazepam effect under WIN.

Supplementary data: Immunohistochemical confirmation of synapse–specific α-subunit distribution

When the initial project was conducted, technical difficulties prevented immunohistochemical confirmation of the electrophysiological results obtained with the CB1-receptor agonist. The necessary triple stainings have meanwhile become possible. We therefore stained coronal mouse brain sections (50 µm) with antibodies against α1- or α2-subunits, respectively, together with antibodies against the postsynaptic marker gephyrin and the cannabinoid receptor CB1. Colocalization was assessed by analyzing confocal sections (60 µm x 60 µm) sampled randomly in the BLA complex of three animals. Gephyrin clusters colocalized with one of the α-subunits were identified, yielding the total number of GABAergic postsynaptic synapses for this section. In a second step, the apposition of these clusters with CB1-positive terminals was quantified. On average one confocal section contained 69 (normalized to 1000 μm^2) postsynaptic clusters positive for α1-subunits of which 30 (43.7±1.5%, n=10 sections) were apposed to CB1 containing presynaptic terminals. For the α2-subunit, one section contained on average 55 positively stained postsynaptic clusters, of which 22 colocalized with CB1 containing terminals (36.4±1.6%, n=8 sections). α1-$GABA_A$ receptors are therefore more likely to be localized at synapses containing the CB1 receptor (which is in accordance with the electrophysiological data). Yet more data are needed before a definite conclusion can be drawn.

Strikingly, there were obvious differences between the two staining patterns: α2-containing receptor clusters appeared to be smaller on average than α1-containing clusters with the latter forming conspicuously large synapses with the CB1 receptor (Fig. 6a). Furthermore, α2-receptors located at the axon hillock were never observed to colocalize with CB1 receptors, suggesting that these particularly effective inhibitory sites are not subject to cannabinoid modulation in the BLA complex (Fig 6b).

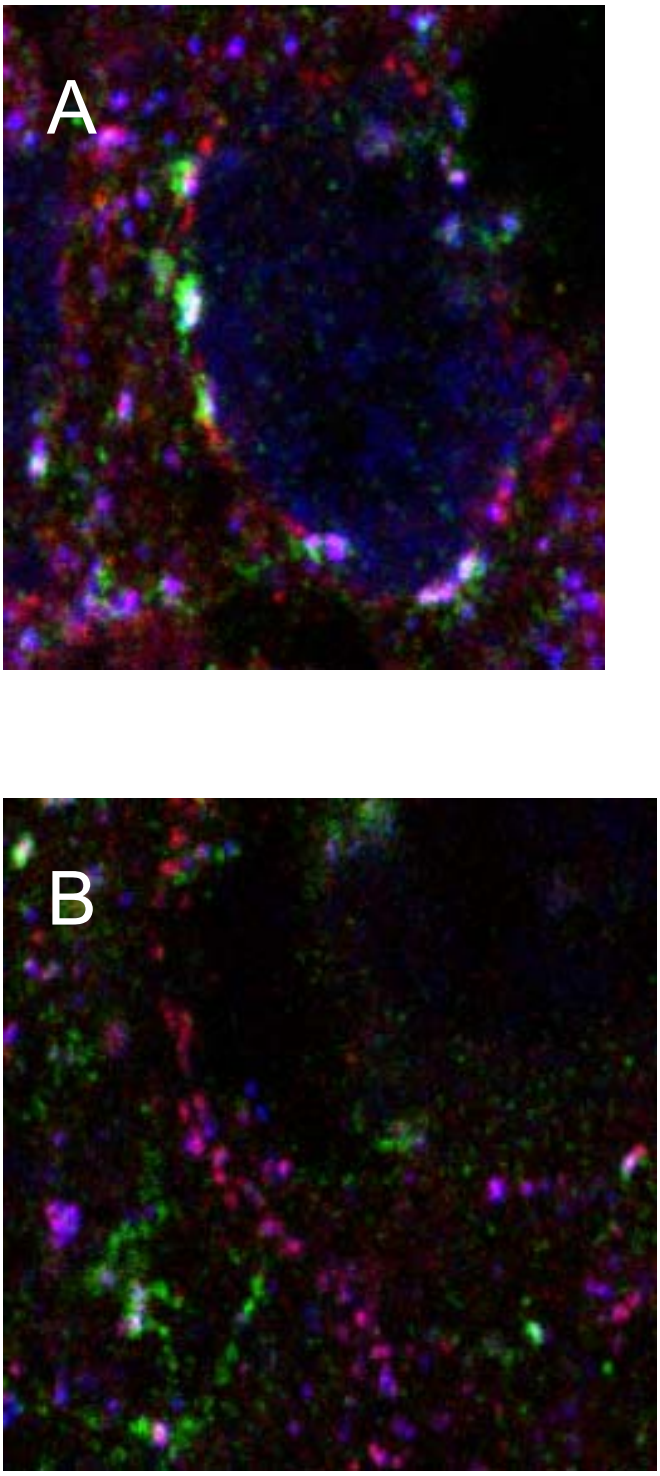


Figure 6. A) Triple immunofluorescence staining for the α1-subunit (red), CB1-receptor (green) and gephyrin (blue) in the basolateral complex of a wildtype mouse, demonstrating colocalization/apposition of CB1 and the postsynaptically located α 1-subunit along the cell soma. B) Same staining for the α2-subunit. Note the prominent expression of this subunit along the axon hillock and the lacking of colocalization with the CB1 protein at this site.

4.2. Discussion

The objective of this work was to study the contribution of $GABA_A$ receptors with different alpha subunits to inhibitory currents in the basolateral (BLA) and central nucleus (CeA) of the amygdala, applying electrophysiological and morphological techniques. The two methods ideally complement each other, as physiological studies allow the revelation of the relative abundance of one subunit compared to another in a distinct nucleus, while immunohistochemical stainings allow for the assessment of the expression level of one subunit across different nuclei. Knock-in mice with diazepam-insensitive α subunits, in particular α1, α2, α1,2 and α1,2,3, were used to address this question. In addition, an attempt was made to identify the $GABA_A$ receptor composition in regard to the α-subunit in synapses formed by CB1 positive interneurons.

The main findings in the present study were

- IPSCs in the basolateral amygdala were mediated both by α2- and α1-$GABA_A$ receptors with α2-$GABA_A$ receptors being predominantly responsible for the diazepam potentiation of inhibitory currents in this area. The contribution of α3-subunit containing receptors was small and could not be statistically resolved.
- IPSCs in the central amygdala were mediated by α2-$GABA_A$ receptors with a minor contribution of α3-subunit containing receptors that could not be statistically resolved.
- Immunohistochemial stainings corroborated these electrophysiological findings on a morphological level.
- The population of CB1-expressing interneurons in the basolateral amygdala signals predominantly through α1-$GABA_A$ receptors; again these results were confirmed by immunohistochemical stainings.

Alpha subunit composition of $GABA_A$ receptors in BLA and CeA

Diazepam acts as a positive allosteric modulator on $GABA_A$ receptors containing either α1,2,3 or α5-subunits together with γ2-subunit, whereas α4- and α6-subunit containing $GABA_A$ receptors do not bind diazepam at the concentrations used here (Wieland et al., 1992). The expression level of the α4-subunit is extremely low in the amygdala and the α6-subunit is only found in the cerebellum (Benke et al., 1997; Pirker et al., 2000), but a high expression of the γ1 subunit was reported in the CeA (Pirker et al., 2000). Hence, most $GABA_A$ receptors in the BLA are expected to bind 'classical' benzodiazepine site ligands such as diazepam, while the occurrence of the γ1-subunit in the CeA complicates an interpretation.

The most pronounced finding, both functionally and morphologically, from the subunit mapping studies is the predominant presence of α2-subunit containing $GABA_A$ receptors in the central amygdala and the concomitant failure to detect α1-subunits in this region. While one cannot rule out the presence of small amounts of α1-subunits below our detection threshold, its levels in the central amygdala are certainly very low. This in contrast to findings in the rat (Pirker et al., 2000), where α1-subunit containing $GABA_A$ receptors were found in the central nucleus of the amygdala. The absence of α1-subunit immunoreactivity from the central nucleus was not due to immature circuitry, as it was still observed in two months old mice.

mIPSCs recorded in BLA and CeA differ in their kinetics

Rise time and decay time constant were distinctly higher in mIPSCs recorded in CeA compared to those recorded in BLA irrespective of the mice genotype used. These altered kinetics might be caused by an enhanced amount of filtering for example due to more elaborate dendritic branching pattern in CeA cells. However, cells in the CeA having about twice the input resistance and roughly the same membrane time constant should possess only half the surface area of BLA cells, thus are rather compact cells. The different kinetics of the receptors might account for the phenomenon, as the α2-$GABA_A$ receptors have been reported to give rise to currents with slower kinetics than receptors containing α1-subunits (Vicini et al., 2001). This together with the lack of α1-

$GABA_A$ receptors in the CeA offers a good explanation for the observed difference in mIPSC kinetics between the two nuclei.

The GABAergic inhibitory system is not impaired by the point mutation

The point mutations at histidine 101 (α1, α2) and histidine 126 (α3) resulted in a slight reduction of the sensitivity of the receptors to GABA when tested under steady-state conditions with heterologously expressed receptors in HEK 293 cells (Benson et al., 1998). These differences were not apparent under conditions of fast agonist transients, as they occur during synaptic release. Miniature IPSCs had similar mean amplitudes and kinetics in wt mice and in mice carrying single α-subunit point mutations in the BLA and in the CeA. In the CeA, where almost all of the inhibitory currents are carried by α2-subunit containing $GABA_A$ receptors, the absence of alterations in mIPSC properties in mice carrying the α2-subunit point mutation strongly argues against major changes in receptor affinity and kinetics. The stimulus strengths to evoke IPSCs of comparable amplitude were also not significantly different in all five strains of mice tested. By abolishing benzodiazepine binding, these point mutations affect a site for which no clear endogenous ligand has been identified. The immunohistochemical results in this study, as well as earlier ones have not revealed differences in receptor distribution patterns (Low et al., 2000; Rudolph et al., 1999) between wild type mice and mice carrying α-subunit point mutations. As also shown by (Fagiolini et al., 2004), $GABA_A$ receptors containing point mutated α-subunits are expressed and assembled into functional receptors, which are not detectably different from receptors containing wild type α-subunits in their distribution and response to synaptically released GABA.

Potentiation of IPSC amplitude by diazepam is different in BLA and CeA

Diazepam potentiation of the IPSC amplitude in wt mice was about twice as high in BLA compared to CeA (78%vs 40%). The degree of potentiation of diazepam sensitive receptors depends on their degree of saturation during synaptic transmission (Frerking et al., 1995). In fact, variability in the response to diazepam by different types of inhibitory synapses have been attributed to different degrees of receptor occupancy

(Hajos et al., 2000; Nusser et al., 1997). The prominent potentiation under diazepam in the BLA complex made it unlikely that a large fraction of $GABA_A$ receptors was saturated in this nucleus. Further support for this conclusion comes from the increase in mIPSC amplitude observed under diazepam indicating that single quanta are also not saturating at these $GABA_A$ receptors and that the observed increase in amplitude under diazepam is not due to spillover onto extrasynaptic receptors. Thus, the smaller diazepam-induced increase in IPSC amplitude in CeA might be due to a higher degree of saturation of the responsive receptors, or the presence of a population of receptors that are saturated together with non-saturated receptors. The cumulative probability plot of the peak mIPSC amplitudes before and after diazepam should reveal the nature of the receptors as a complete shift to the right indicates the occurrence of a uniformly non-saturated receptor population while a partial one is indicative of the co-existence of a saturated and a non-saturated receptor population (Perrais and Ropert, 1999). Although the two curves differ only slightly for small amplitudes, there is an overall shift to the right observable, suggesting the existence of a homogeneously unsaturated population. An alternative explanation for the difference in the amount of diazepam potentiation between the two nuclei lies in diazepam-insensitive γ1-containing $GABA_A$ receptors (Pirker et al., 2000). Such a receptor would contribute to IPSCs under baseline conditions, yet not to the diazepam-potentiated signal. Finally, (bicuculline-insensitive) $GABA_C$ receptors were described in the rat CeA by Delaney and Sah (Delaney and Sah, 1999; Delaney and Sah, 2001). Since diazepam decreases $GABA_C$-mediated IPSCs, their presence could contribute to a lesser IPSC potentiation in this nucleus. However, we failed to detect a bicuculline-resistant (1 μM) chloride current, arguing against the presence of a glycine-, or $GABA_C$-receptor mediated, diazepam-insensitive current.

Given the existence of a homogenous non-saturated α2-$GABA_A$ receptor population in the CeA, α2- $GABA_A$ receptors might be less potentiated than those containing α1-subunits. In this case the proportion of the current mediated by α2-subunit containing $GABA_A$ receptors would be underestimated using purely the loss of peak amplitude potentiation as a measure.

In contrast to the immunohistochemical data showing a medium level of α3-subunit expression in BLA and CeA, we could only detect a modest contribution of this subunit to the inhibitory signals in both BLA and CeA. An explanation might lie in the extrasynaptic localisation of α3-containing receptors as observed in the inferior olivary

neurons (Fritschy and Brunig, 2003), resulting in an altered benzodiazepine response of this point mutation.

Diazepam affects decay time constants of IPSCs in an inconsistent way

Diazepam also causes an increase in the decay time constant of evoked and miniature IPSCs and this increase in decay time constant is not affected by the degree of receptor saturation. The effect of the different point mutations on this phenomenon was too modest and varied too strongly as to allow for a quantitative interpretation. Diazepam-sensitive α5-subunits, highest expressed in the basolateral nucleus and to a lesser degree in the lateral and CeA, and perhaps α3-subunits might be responsible for this inconsistency. They are mainly found extrasynaptically (Fritschy and Brunig, 2003) and thus are not expected to contribute to the peak amplitude of an evoked IPSC, but to shape the decay phase of the signal.

CB1-carrying interneurons signal largely through alpha1 $GABA_A$ receptors

There is morphological evidence for $GABA_A$ receptor specificity at certain types of interneuronal connections, in particular hippocampal CCK positive and PV positive basket cells were shown to signal mainly through α2-GABAA respectively a α1-$GABA_A$ receptors (Klausberger et al., 2002; Nyiri et al., 2001). Large CCK positive inhibitory interneurons are also found in the BLA complex (Mascagni and McDonald, 2003). Depression of synaptic release from CB1 receptor carrying inhibitory terminals reduced mainly the contribution of α1-subunit containing $GABA_A$ receptors to the remaining IPSC, suggesting that in the amygdala a large fraction of CB1 carrying synapses contain α1-subunits, and only a smaller fraction is associated with α2-subunits. Our immunohistochemical stainings, though preliminary, support the electrophysiological findings, suggesting a different cannabinoid modulation of inhibition in amygdala and hippocampus.

Conclusion

Intra-amygdala application of benzodiazepines is effective in producing anxiolysis (Nagy et al., 1979; Shibata et al., 1982) and the anxiolytic effects of systemic benzodiazepine application can be blocked by focal injection of the specific antagonist flumazenil into the amygdala(Hodges and Green, 1987). These effects were found to be restricted to the BLA complex in several studies (Hodges and Green, 1987; Niehoff and Kuhar, 1983; Thomas et al., 1985). Together with the behavioral data from the point mutated mice (Low et al., 2000) this indicates that GABAergic currents in the BLA complex mediated by α2-$GABA_A$ receptors and their modulation by diazepam are crucial for anxiolysis. Diazepam potentiation of the sizeable pool of $GABA_A$ receptors containing α1-subunits in the BLA complex, however, does not lead to anxiolysis (Rudolph et al., 1999). This means that either interneurons responsible for the modulation of anxiety do not signal through α1- $GABA_A$ receptors, or that the potentiation of these receptors has a profoundly different effect on the target neurons. Our findings provide evidence for the former concept as our data suggest that CB1-positive synapses are predominantly endowed with α1-$GABA_A$ receptors with CB1-receptors playing a crucial role in fear extinction (Marsicano et al., 2002).

Within this framework, activation of a population that predominantly or exclusively signals through α2-subunit containing $GABA_A$ receptors would control anxiety, whereas another, CB1 carrying population of interneurons signaling predominantly through α1-subunit containing $GABA_A$ receptors exerts among other things influence over the extinction of aversive memories

5 Project 2: Dopamine Facilitates Amygdala Function Through the Inhibition of a Network of Specialized Feedforward Interneurons

5.1. Results

Morphological and physiological characterization of paracapsular intercalated cells

GAD-GFP mice (Tamamaki et al., 2003) express green fluorescent protein (GFP) under the control of the promotor for GAD67, a key enzyme for the synthesis of γ-aminobutyric acid (GABA). In the amygdala, two subgroups of densely packed interneurons can be identified, one located along the lateral border of the BLA next to the external capsule (*) and the other along the medial border (**) between the BLA and central amygdala (Fig. 1A, B, C). They are occasionally linked by a cell band stretching across the BLA (Fig. 1B, downward arrow). Consistent with location and morphology, these neurons are collectively classified as paracapsular intercalated cells (pcs) (Millhouse, 1986) with a lateral (lpcs) and a medial (mpcs) subdivision. Subsets of interneurons in the BLA were immunopositive for typical interneuron markers (calbindin, calretinin, parvalbumin, NPY, cholecystokinin, somatostatin), none of which labeled pcs (data not shown).

In comparison to other interneurons of the BLA, pcs exhibited broader spikes, less pronounced afterhyperpolarization and greater spike frequency adaptation. These features were especially prominent in lpcs, while the firing pattern of mpcs more closely resembled the one observed in other BLA interneurons (Fig. 1D). The passive membrane properties of pcs were characteristic of small neurons, with the lpcs possessing even higher input resistance and lower whole-cell capacitance than mpcs (Table 1).

Table 1. Electrophysiological properties of:
lateral (lpc) and medial (mpc) paracapsular intercalated cells and
interneurons within the basolateral amygdala (IN BLA).

	lpc			mpc			IN BLA		
	mean	sem	n	mean	sem	n	mean	sem	n
Parameter									
RMP (mV)	-78	1.3	44	-78	1.6	36	-77	2.8	25
Rin (MOhm)	865	31	44	657	49	36	341	37	25
Cap (pF)	57.1	1.2	44	59	3.1	36	93	7.2	25
peak f (Hz)	19.9	1.2	43	24.8	1.9	36	45.4	4	25
sustained f (Hz)	14.8	0.35	43	19.4	1	36	39.5	0.6	25
f adapt (rel)	0.75	0.03	43	0.78	0.05	36	0.8	0.02	25
hw first (ms)	1.7	0.032	43	1.2	0.034	36	1.2	0.09	25
hw sust (ms)	2.85	0.053	43	1.9	0.093	36	1.37	0.037	25
AP first (mV)	67	1.4	43	68	1.4	36	70	1.9	25
AP sust (mV)	51	0.8	43	54	1.2	36	60	0.5	25

We measured: resting membrane potential (RMP), input resistance (Rin), whole cell capacitance (Cap), peak firing frequency - first three action potentials (peak f), sustained firing frequency - last four action potentials (sustained f), frequency adaptation (sustained frequency divided by the peak frequency), action potential half-width of the first action potential (hw first), action potential half-width of the last action potential (hw sust), Amplitude of the action potential from the beginning of the upstroke to the peak for the first action potential (AP first) and for the last action potential (AP sust). Average values (mean), standard error of the mean (sem) and the number of recordings (n) are given.

Dopaminergic modulation of pcs

Tyrosine hydroxylase positive fibers were found within lpc- and mpc-clusters in GAD-GFP mice (Fig. 2A) in accordance with previous data obtained in the rat (Asan, 1998). To assess the function of this innervation, we first studied the direct effects of DA on pcs. Bath application of DA (35-70 μM) caused a large hyperpolarization of the resting membrane potential of pcs from -76.6± 0.9 mV to -84.6±1.0 mV (n=23, $p<0.001$) (Fig. 2B), which was reversible upon washout. No significant difference in the amount of hyperpolarization was observed between lpcs and mpcs (lpc: n=13, -8.7±0.9 mV; mpc: n=10, -7.1±1.3 mV, $p>0.3$). This DA-mediated hyperpolarization is unique among inhibitory cells in the amygdala, as classical BLA interneurons were depolarized by DA (n=7, -75.1± 2.2 mV to –71.7±1.9), consistent with previous results (Kroner et al., 2005; Loretan et al., 2004).

The hyperpolarization was accompanied by a significant drop in the likelihood of action potential firing in response to evoked EPSPs after DA application (51±9% before DA vs. 12±7% after DA, n=4, $p<0.05$) (Fig. 2B1), indicating that these cells are less excitable under DA.

To verify that DA directly affected membrane potential and excitability of pcs, we repeated the experiments under complete block of ionotropic glutamatergic and GABAergic synaptic transmission (3 mM kynurenate, 100 μM picrotoxin) while evoking action potentials via somatic current injections (Fig. 2C1). Under these conditions, the membrane potential was hyperpolarized from -74.4±1.8 mV to -82.2±1.6 mV (n=8, $p<0.01$), accompanied by a reduction of the input resistance (886±35 MΩ to 660±48 MΩ, n=8, $p<0.01$) and of evoked spikes from 6.5±1.1 to 1.6±0.6 per trial (n=8, $p<0.01$) (Fig. 2C2-C4). Five experiments were also done in the presence of the $GABA_B$ receptor antagonist CGP55845 (5 μM), which did not affect the amount of hyperpolarization observed (data not shown). As synaptic activity was not necessary for the hyperpolarization, we concluded that DA attenuates the excitability of pcs via a direct postsynaptic effect by increasing a hyperpolarizing conductance.

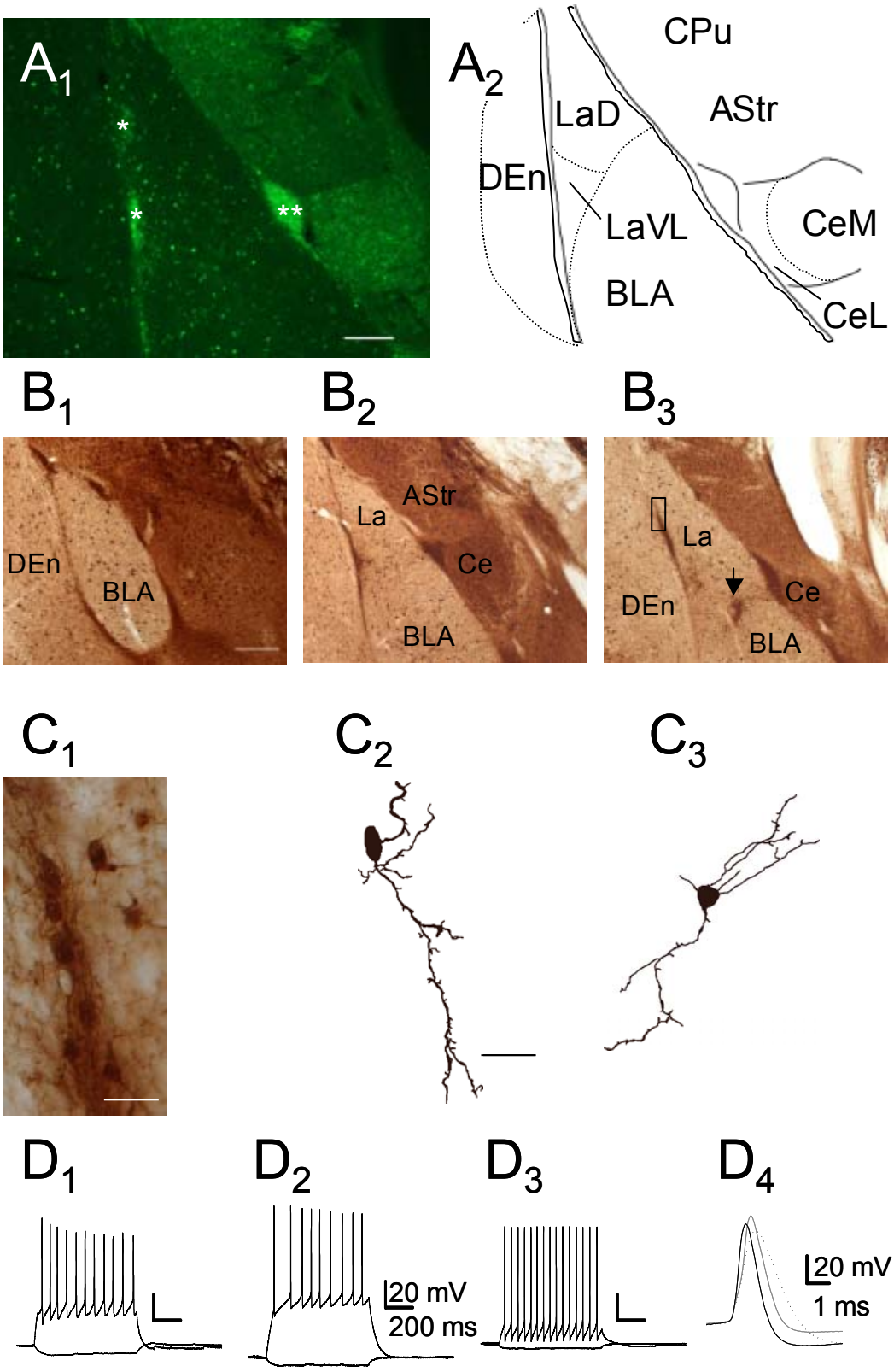

A1
A2
CPu
AStr
LaD
DEn
LaVL
CeM
BLA
CeL
B1
B2
B3
DEn
BLA
La
AStr
Ce
BLA
La
DEn
Ce
BLA
C1
C2
C3
D1
D2
D3
D4
20 mV
200 ms
20 mV
1 ms

Figure 1 ***Location, morphology and basic physiology of paracapsular intercalated cells***

(A) Paracapsular intercalated cells (pcs) are located at both borders of the lateral and basolateral amygdala. (A1) They can be identified as clusters (* lateral pcs (lpcs) and ** medial pcs (mpcs)) of densely packed fluorescent cells in acute brain slices from GAD-GFP mice. Compare with the more sparsely occurring interneurons within the BLA. Scale bar: 200 µm. (A2) Schematic of A1, with: LaD, lateral amygdala dorsal nucleus; LaVL, lateral amygdala venterolateral nucleus; BLA, basolateral amygdala; CPu, caudate putamen; Astr, amygdalostriatal transition area; CeM, medial division of the central nucleus; CeL lateral division of the central nucleus; DEn, dorsal endopiriform nucleus.
(B) DAB staining against GFP in fixated slices obtained from GAD-GFP mice, GABAergic cells are dark brown. B1) Rostral section: Intercalated cells form a continuous shell around the BLA. (B2) Intermediate section: pcs are found as dense clusters along the external and intermediate capsule. (B3) Caudal section: the two clusters are linked by strands of similarly dense interneurons (downward arrow). Rectangle marks the region enlarged in C1. Scale bar: 200 µm
(C) The pcs are small, sparsely-spined interneurons; lpcs are spindle shaped, whereas mpcs are oval. (C1) Inset from B3, note the dense network of dendrites running in the dorso-ventral direction along and within the external capsule. (C2) Biocytin filled lpc. (C3) Biocytin filled mpc. Scale bar: 20 µm.
(D) Spiking patterns of GABAergic cells. (D1) Response of an lpc to somatic current injections of –10 pA and 50 pA. (D2) Response of an mpc to –10 pA and 60 pA current injection. (D3) Typical interneuron within the BLA, response to –10 pA and 90 pA current injection. (D4) Enlarged plot of the first action potential from the three cell types – lpcs (dotted line) mpcs (gray line) and BLA interneuron (black line).

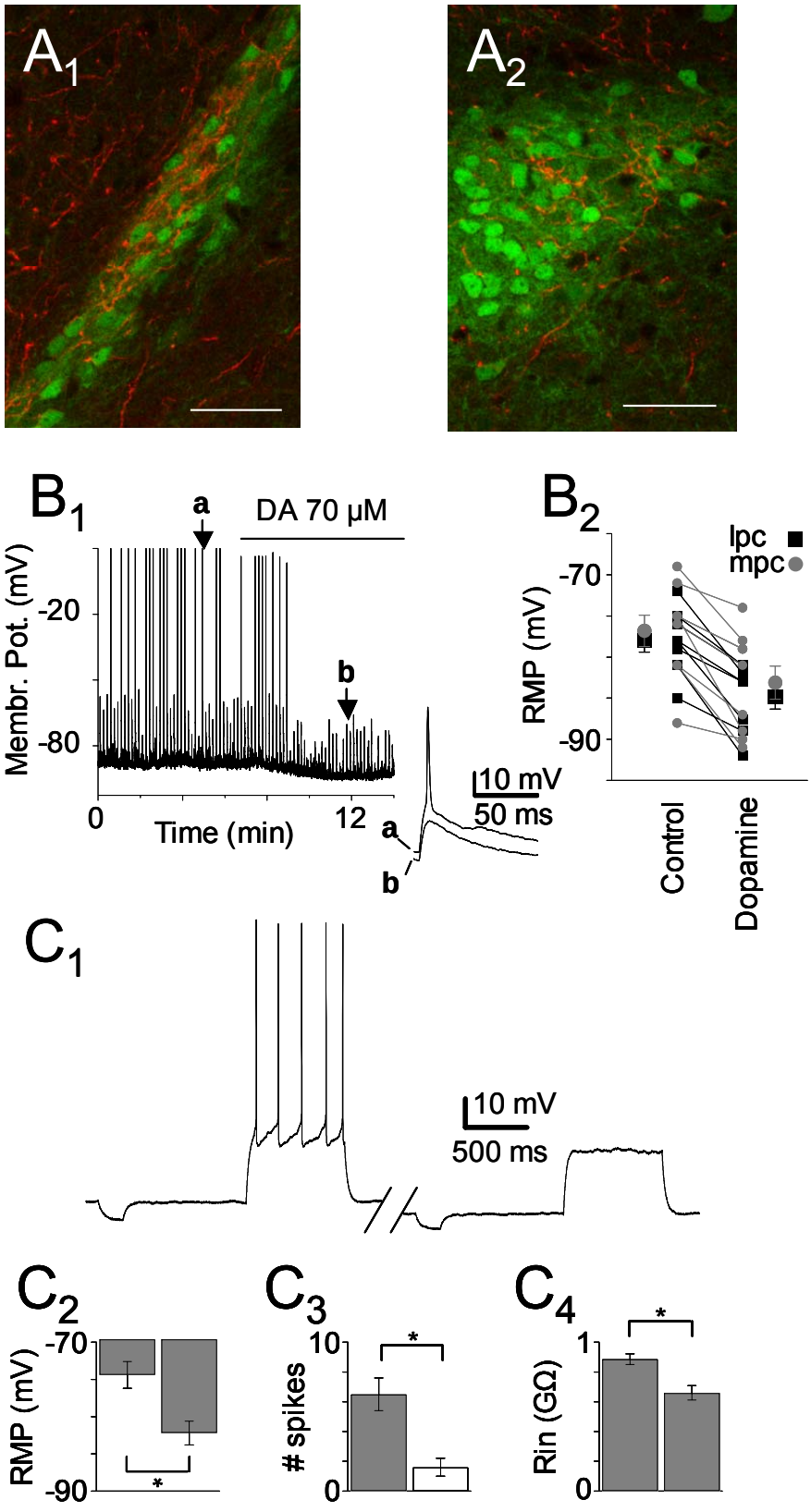

A1
A2
B1
a
DA 70 µM
b
Membr. Pot. (mV)
-20
-80
0
Time (min)
12
10 mV
50 ms
B2
lpc
mpc
RMP (mV)
-70
-90
Control
Dopamine
C1
10 mV
500 ms
C2
RMP (mV)
-70
-90
*
C3
spikes
10
0
*
C4
Rin (GΩ)
1
0
*

Figure 2 ***DA hyperpolarizes pcs and reduces their excitability***

(A) Confocal images of slices from GAD-GFP mice stained against tyrosine hydroxilase. Tyrosine hydroxylase positive fibers (red) can be found within the green fluorescent clusters of (A1) lpcs and (A2) mpcs. Scale bar: 40 μm.
(B) Dopamine hyperpolarizes and inhibits pcs. (B1) Current-clamp recording of an lpc with a stimulus electrode placed in the external capsule. The resulting EPSP elicits an action potential in about 50% of the cases. Application of dopamine (DA, indicated by the horizontal line) results in a hyperpolarization and loss of spiking. Inset shows sample traces before (a) and after (b) the application of DA. Note that the EPSP size is not obviously reduced. (B2) Individual recordings and summary of resting membrane potentials (RMP) in lpcs (black squares) and mpcs (gray dots), in control conditions and after DA.
(C) DA affects pcs directly. (C1) Response of an lpc to somatic current injections (-10 pA and 40 pA, respectively) before and after the application of DA (70 μM) in the presence of kynurenic acid (3 mM) and picrotoxin (100 μM). The cell is hyperpolarized and its input resistance is reduced resulting in a loss of spike generation. (C2) Resting membrane potential (RMP) in all pcs before and after DA. (C3) The number of spikes due to the somatic current injection before and after DA. (C4) Input resistance of all pcs at baseline and after application of DA.

Dopamine activates a GIRK channel in pcs

To characterize further the conductance underlying the hyperpolarization, we dialyzed pcs with a CsCl-containing internal solution, and voltage-clamped them at -60 mV. Under these conditions, no change in holding current was observed after application of DA (n=4), whereas in pcs filled with a K-gluconate-based internal solution and voltage-clamped at -40 mV (n=4) an outward current was induced (data not shown). These data argue against a chloride conductance underlying the DA effect. Voltage ramps (800 ms) from –100 mV to –60 mV applied before and after the addition of DA, revealed an inward rectification and a reversal potential of -84.1±0.93 mV (n=4) for the induced current (Fig 3A). In addition, dialyzing the cells for 25 min prior to recording with the antagonist of G protein activation GDP-βS (1 mM in the recording electrode) prevented hyperpolarization under DA and led to a slight depolarization instead (Fig. 3B). These data provide good evidence for the opening of G protein coupled inward rectifier potassium (GIRK) channels in pcs under DA.

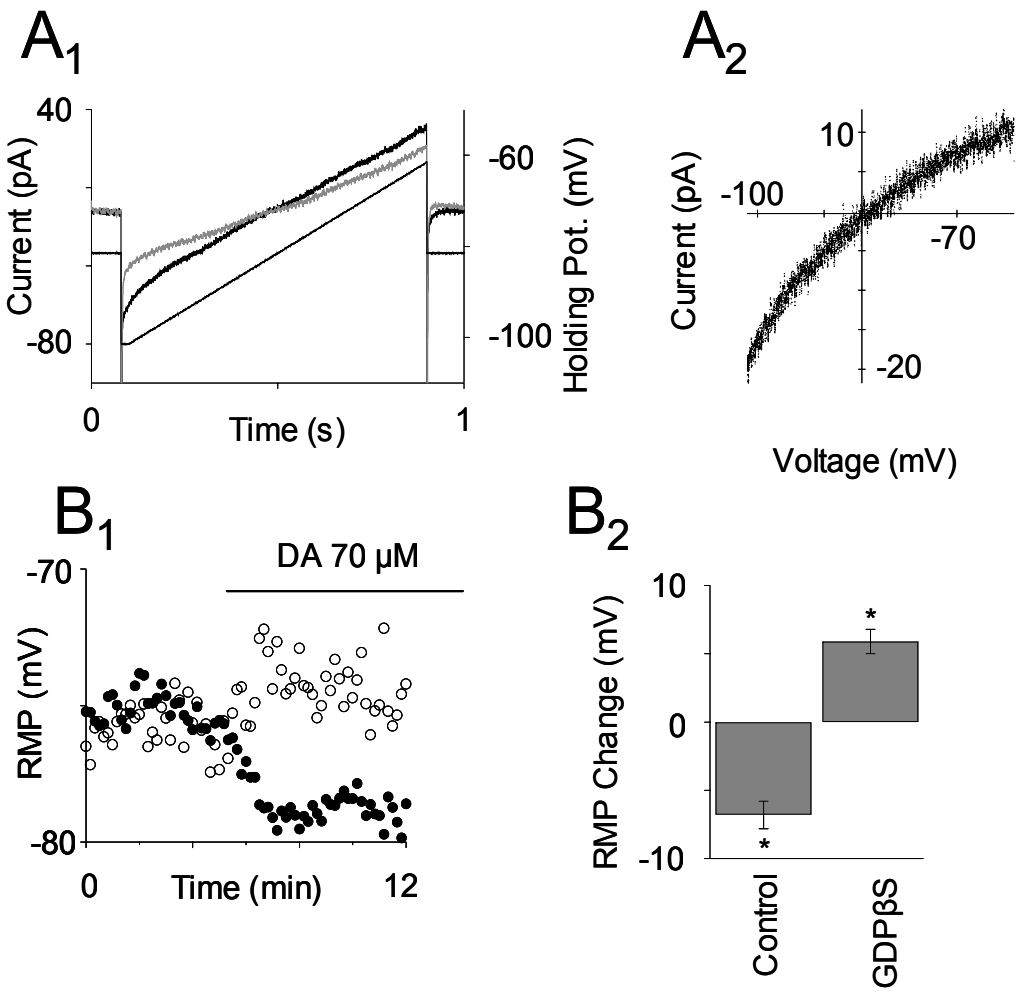


Figure 3 ***DA increases a G-protein dependent inward rectifying potassium conductance in pcs***.

(A1) Cells were voltage clamped with a Kgluconate-based internal solution. The current response (left scale) of a pc to the application of a voltage ramp (thin black line, right scale) before (gray trace) and after (black trace) the application of DA, (average of 10-20 traces). (A2) The resulting current-voltage relationship. The current reverses at -84 mV and shows inward rectification.
(B) The pc hyperpolarization is dependent on G-protein activation. (B1) Recording of the resting membrane potential (RMP) in two pcs. A control neuron dialyzed for 20 min with normal internal solution (filled circles) and a cell that was dialyzed for 20 min with 1 mM GDPβS in the internal solution (open circles). DA no longer hyperpolarized the GDPβS treated cell. (B2) Summary of the DA effect on RMP in four cases each.

D_1 receptors largely mediate the dopaminergic effect

The relatively selective D_1 receptor agonist dihydrexidine, but not the highly selective D_2 agonist quinpirole, mimicked the effects of dopamine application in both lpcs and mpcs (Fig. 4A). In lpcs (n=7), dihydrexidine (10 μM) caused a hyperpolarization (-74.8 ±1.0 mV to -79.6±1.0 mV, $p<0.01$), a concomitant loss of input resistance (791±43 MΩ to 545±67 MΩ, $p<0.05$), and a decrease in the number of evoked action potentials (9.2±2.0 to 4.0±1.6, $p<0.05$). Consistently, DA combined with the D_2 receptor antagonist sulpiride (20 μM) hyperpolarized the resting membrane potential (-73.0±1 mV to -80.2 ±2.3 mV, n=5, $p<0.05$), whereas quinpirole (20 μM) as well as dopamine together with the D_1 receptor antagonist SCH 23390 (10 μM) led to a depolarization (-73.4± 0.6 mV to -69.6±1.2 mV, n=5, $p<0.01$ and -73.3 ±1.0 mV to –71.5±0.9 mV, n=4, $p<0.01$) (Fig. 4B). Essentially the same results were obtained for mpcs with the exception of a differing sensitivity to D_2 receptor activation (Fig 4B). DA combined with the D_2 receptor antagonist sulpiride (20 μM) did not mimic the effect of the D_1 receptor agonist, but resulted in a depolarization in five out of seven cells (-73.4±0.5 mV to -70.4±1.4 mV, $p>0.05$). The D_1 receptor agonist dihydrexidine (10 μM) by itself reproduced the dopaminergic effect, demonstrated by a significant hyperpolarization of membrane potential, decreased input resistance and reduced number of evoked spikes (n=4, –74.5 mV±0.9 to –78.3±0.8 mV, $P<0.01$; 1010±66 MΩ to 751±116 MΩ, $P<0.05$; 8.8±1.7 to 5.0±1.2, $P<0.05$). Quinpirole (20 μM) and the combination of the D_1 receptor antagonist SCH 23390 (10 μM) with dopamine caused again a depolarization (-72.5±0.5 mV to -71.8±0.5, n=5, $P>0.05$, and -72.9±0.7mV to -69.8±1.3mV, n=5, $p<0.05$).

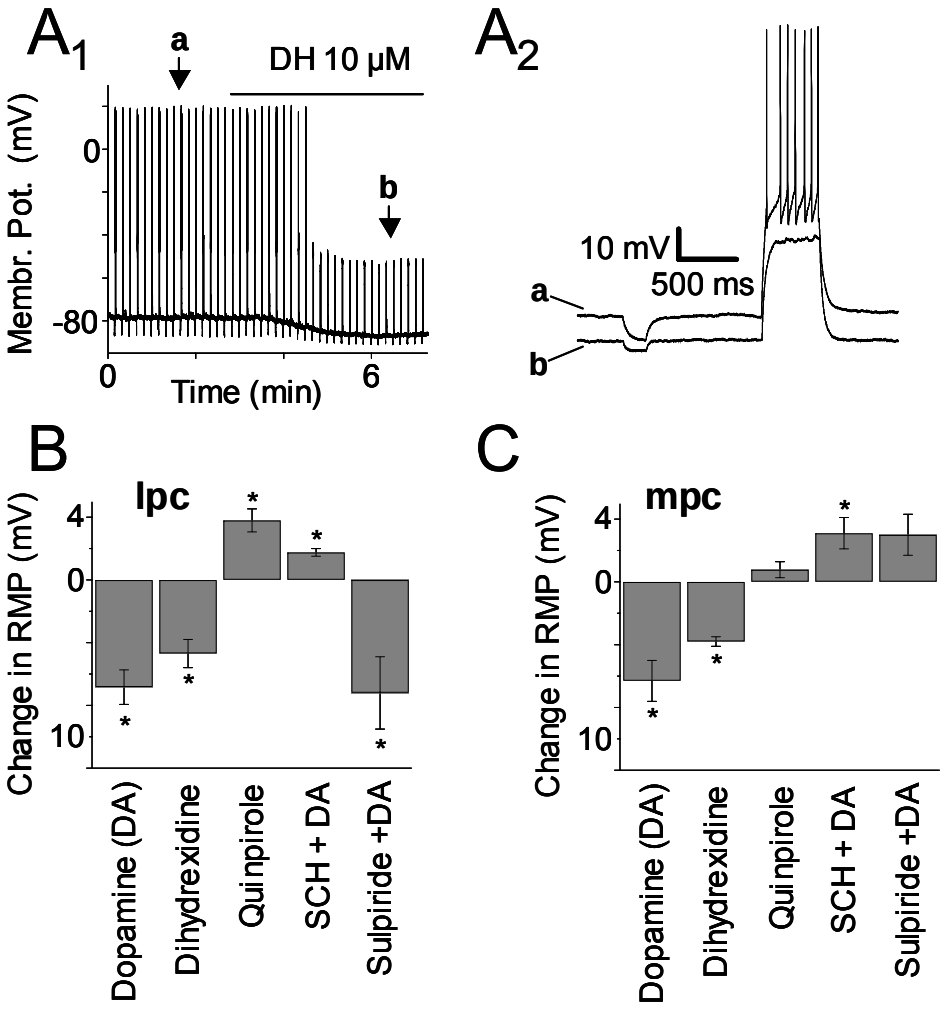


Figure 4 ***D_1 receptor activation on pcs mimics the DA effect.***

(A) The D_1 receptor agonist dihydrexidine (DH) hyperpolarizes and inhibits lpcs. (A1) Current-clamp recording of a lpc under kynurenic acid (3 mM) and picrotoxin (100 µM). Somatic current injections of –10 pA and 50 pA were applied once every 10s. The neuron fired a series of action potentials as a result of the positive current injection (A2, top trace). Application of DH (A1, horizontal line) caused a loss of input resistance and a hyperpolarization and the cell no longer fired action potentials (A2, bottom trace). (B) Summary of the pharmacological effects on lpc resting membrane potential (RMP). DA, the D1 agonist dihydrexidine and DA in the presence of the D2 antagonist sulpiride evoke a hyperpolarizing response, whereas the D2 agonist quinpirole and DA in the presence of the D1 receptor antagonist SCH 23390 evoke a depolarizing response. (C) In mpcs the response to D1 activation is the same, however the D2 agonist quinpirole does not depolarize mpcs and the D2 antagonist sulpiride prevents DA-induced hyperpolarization.

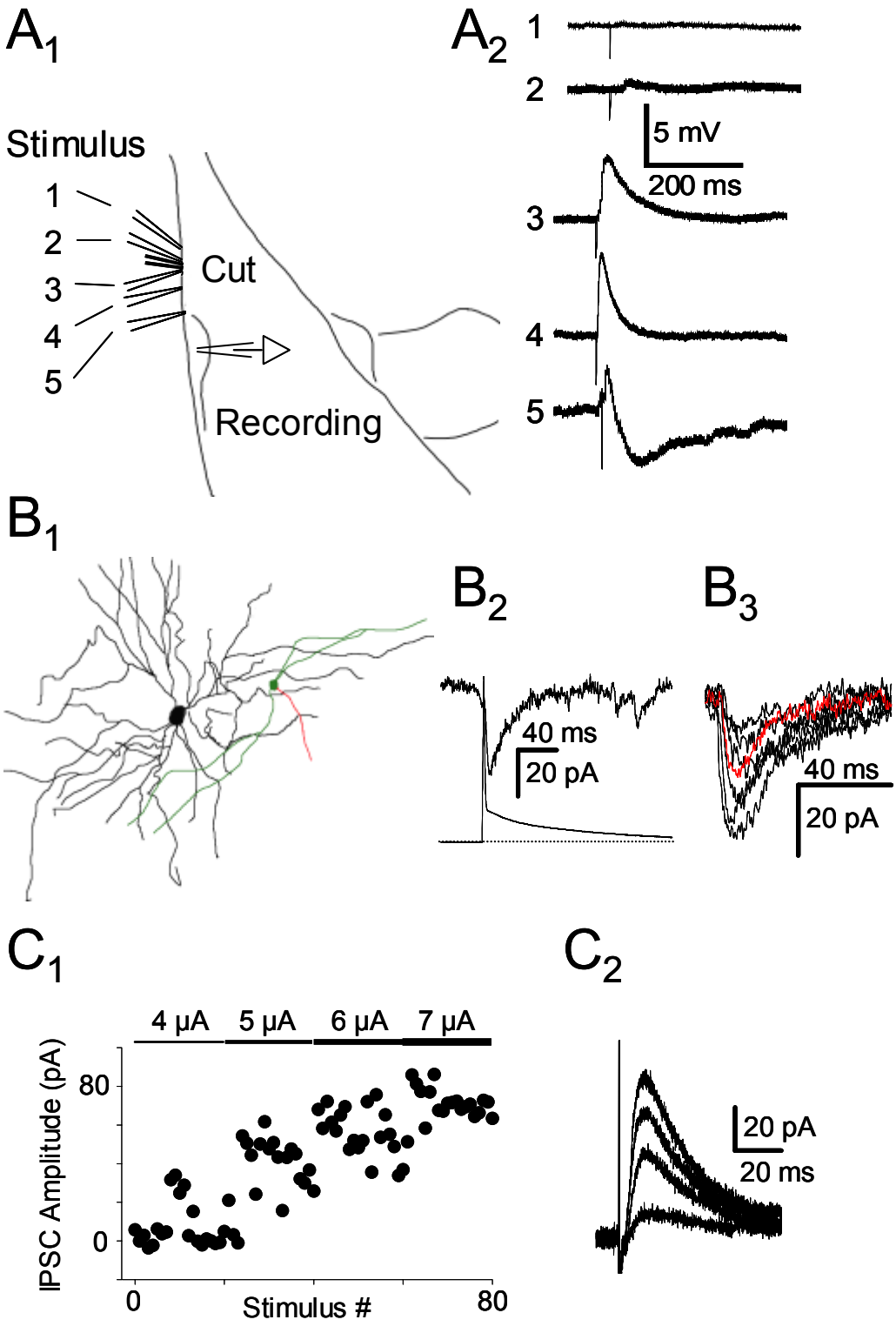
Input and output characteristics of lpc

To understand better the organization of the whole pc network and to assess the impact of DA on its function, we next studied the connectivity of the lpcs.

We stimulated fibers of the external capsule and recorded from identified lpcs (Fig 5A) to determine whether they are innervated by cortical afferents similar to their medial counterparts. Such stimulation evoked excitatory postsynaptic potentials (EPSPs), except when we placed a cut in the external capsule between the recording and the stimulation site (Fig. 5A) (n=4). Although distal stimulation evoked primarily EPSPs, either increased stimulation intensity or more proximal stimulation also recruited IPSPs. These findings show that lpcs receive excitatory cortical innervation, and provide lateral inhibition to other lpcs as has been shown for mpcs (Royer et al., 1999). Individual lpcs were filled with biocytin, revealing sparsely spine-carrying dendrites (a morphology reminiscent of mpcs (Millhouse, 1986; Royer et al., 1999) that for the most part ran within the external capsule, and were intertwined with its fibers (Fig. 1C). Axonal arborizations were observed branching within the lpc cluster, infrequently giving off collaterals into the BLA.

To confirm a functional connectivity between lpcs and their targets in the BLA, we obtained somatic dual whole-cell recordings from 7 lpc-BLA principal cell pairs under block of excitatory synaptic transmission (Fig. 5B2). BLA principal cells were patched with pipettes containing a CsCl solution in order to improve the signal to noise ratio for the recordings. The amplitude of postsynaptic IPSCs in principal cells resulting from single presynaptic spikes evoked by current injection into lpcs averaged 16±1.7 pA. Rise time and decay time constants of the IPSCs were 5.0±0.7 ms and 46.8±13.1 ms, respectively. The small amplitude and slow kinetics suggest dendritic, rather than somatic synaptic contacts, consistent with the distal axo-dendritic contact found in the biocytin-filled cell pair (Fig. 5B1)

Increasing the stimulation current through an extracellular stimulus electrode placed within the lpc cluster resulted in a slowly growing inhibitory response in BLA principal cells (Fig. 5C). This gradual recruitment of inhibition indicates that a given BLA principal cell receives afferents from several lpcs in a cluster.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1
2
3
4
5
Cut
Recording
A2
1
2
5 mV
200 ms
3
4
5
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40 ms
20 pA
B3
40 ms
20 pA
C1
4 µA
5 µA
6 µA
7 µA
IPSC Amplitude (pA)
80
0
0
Stimulus #
80
C2
20 pA
20 ms

Figure 5 ***The lpcs receive excitatory cortical input and send inhibitory efferents to the BLA.***

(A) The response of a lpc to stimulation of the external capsule at different sites. (A1) Schematic drawing of the position of the recorded cell and the different positions of the stimulus electrode. The electrode was sequentially positioned at the numbered sites and an identical stimulus was applied. (A2) Current clamp recordings from the lpc. Distal to a selective cut through the external capsule and adjacent cortex no postsynaptic response could be evoked (1,2). Immediately proximal to the cut the same stimulus strengths evoked an EPSP (3). Moving closer to the recorded cell with the stimulus electrode results in larger EPSPs and the additional recruitment of IPSPs (4, 5). Traces are averages of 8-10 individual responses under the same conditions.

(B) Paired recording between an lpc and a BLA projection neuron. The postsynaptic cell was dialyzed with a high chloride containing solution and therefore shows an inward current, excitatory synaptic transmission was blocked with kynurenic acid (3 mM). (B1) Both cells were filled with biocytin. The dendritic tree of the BLA projection cell is shown in black, the dendrites of the lpc are shown in green and the axon of the lpc is shown in red. (B2) Average (20 traces) monosynaptic IPSC (top trace) in the BLA principal cell as a response to lpc action potential firing (bottom trace) (B3) Overlay of individual traces showing the considerable trial-to-trial variability; in red an averaged response.

(C) Individual BLA-principal cells are connected to several lpcs. (C1) Plot of IPSC amplitude in a BLA-principal cell as a function of increasing extracellular stimulations in the nearby lpc cluster. The current applied to the stimulation electrode was maintained for the times indicated by the horizontal lines and ten traces were gathered at each level. (C2) Average traces at the different stimulus strengths. The postsynaptic cell was patched with a low chloride containing solution and held at -40mV.

DA reduces feedforward inhibition into the BLA and CeA

To study the overall effect of DA on pc output, extracellular stimulus electrodes were placed in either the lpc cluster or the mpc cluster, activating them either directly or through their excitatory synaptic input, and postsynaptic potentials were recorded in their respective target cells (BLA pyramidal cells (n=4), CeA neurons (n=4)). An inhibitory response could always be evoked in the target cells, whereas an excitatory postsynaptic potential was only observed in two of four cells each for the BLA or the CeA. All target cells in BLA and CeA were depolarized under DA (7.5±1.3 mV, n=4, $p<0.05$ and 6.3±1.4 mV, n=4, $p<0.05$) (Kroner et al., 2005), and this made it necessary to repolarize them to their original membrane potential before assessing the dopaminergic impact. Application of DA strongly reduced the IPSP amplitude both in the CeA neurons (15±9% of control, $p<0.05$) and BLA pyramidal cells (25±16% of control, $p<0.05$) without significantly affecting the EPSP amplitude (94±18% of control, n=4, $p>0.7$, pooled data from BLA and CeA). As has been postulated (Rosenkranz und Grace 2002), the reduced inhibitory output of pcs could also be due to a reduced excitatory input to these cells. We therefore recorded monosynaptic EPSCs in pcs evoked by stimulation of the external capsule (in case of lpcs) and either the intermediate capsule or the BLA (in case of mpcs). In lpcs EPSC amplitudes were not affected by DA (98% ± 8% of control, $p>0.5$, n=4). Contrastingly, in mpcs a considerable decrease in the EPSC amplitude was observed (23± 15% of control, n=6, $p<0.05$) when stimulating either the intermediate capsule (n=3) or the basolateral nucleus (n=3). Thus, in mpcs DA exerted a compound effect that involved a reduced excitatory input onto these cells along with an attenuated intrinsic excitability. Taken together, DA strongly shifts the balance towards excitation in both the BLA and CeA by reducing pc output and depolarizing the target cells.

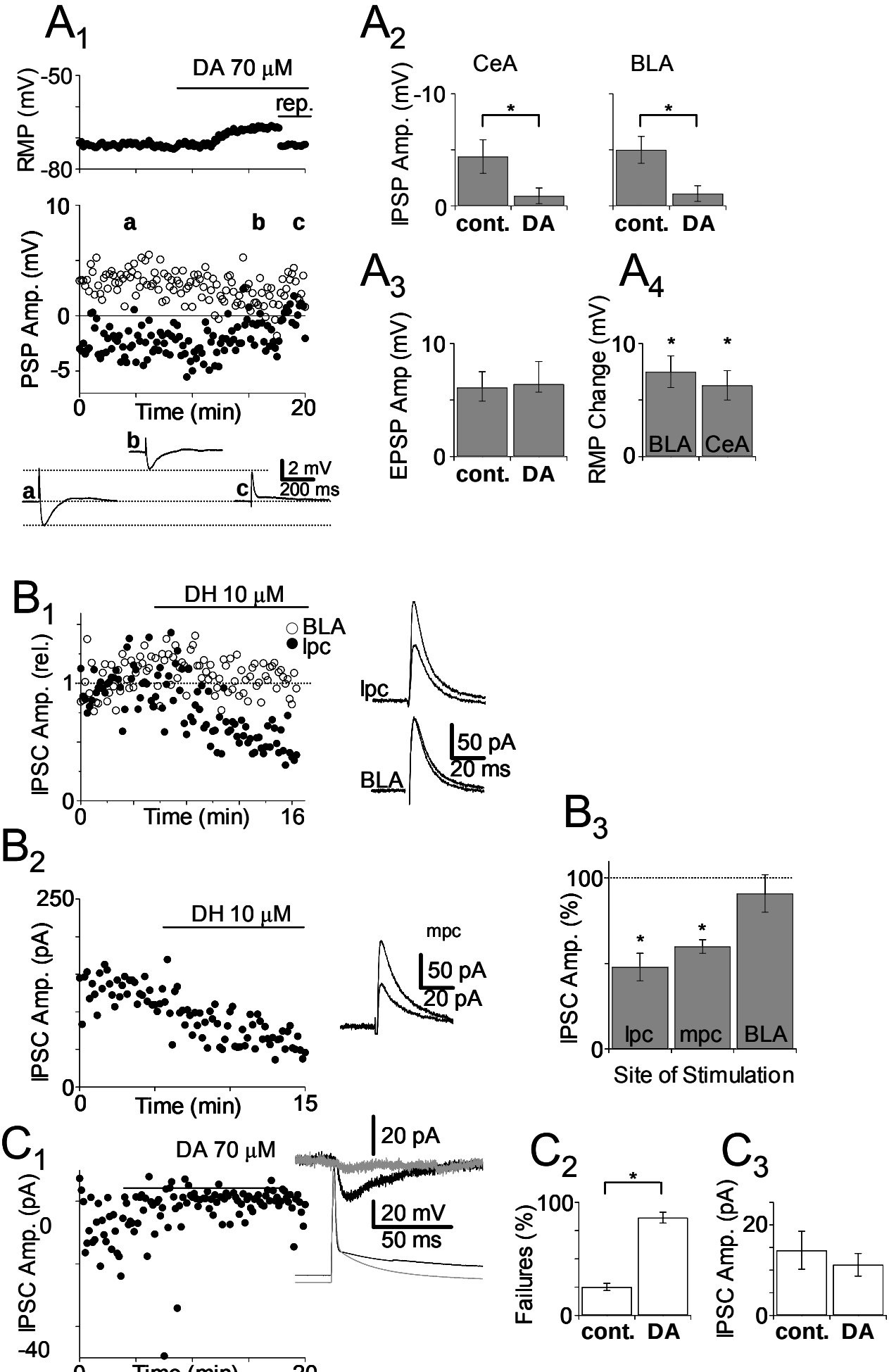
D1 receptor activation selectively reduces pc inhibitory output

However, DA not only affects IPSCs originating from pcs but from BLA interneurons in general (Kroner et al., 2005). To confirm that the recorded inhibitory response predominantly originated from the paracapsular cell clusters, DA was replaced by the D_1 receptor agonist dihydrexidine, as inhibitory transmission generated by other BLA interneurons was shown to be depressed by D_2-, but not D_1-receptor activation (Bissiere

et al., 2003). Target cells in the BLA or CeA were voltage-clamped in the presence of 3 mM kynurenate, and monosynaptic IPSCs were evoked by stimulation of either lpcs or mpcs, respectively. For lpcs, a second stimulus electrode was placed inside the nucleus to evoke control IPSCs from local interneurons. Dihydrexidine reduced the IPSCs produced both by lpc and mpc stimulation (48±8% of control, n=4, $p<0.05$ and 60±4% of control, n=4, $p<0.05$ respectively), and did not affect IPSCs originating from BLA interneurons (IPSC amplitude: 91±11% of control, n=4, $p>0.4$) (Fig. 6A).

In addition to the postsynaptic effect on pcs, DA may also act presynaptically on neurotransmitter release. In three paired recordings from lpcs and BLA principal cells DA hyperpolarized the presynaptic lpcs as expected, however firing was ensured by a sufficiently large somatic current injection (Fig. 6B). In the postsynaptic cell, DA led to an increase in the failure rate of the evoked IPSCs from 25.2±3.2% to 86.0±4.8% (n=3, $p<0.05$), the amplitude of the successes (14.4±4.2pA to 11.2±2.5pA, n=3, $p>0.5$) was not significantly reduced, indicating primarily a decrease in release probability (Fig. 6B).

In summary, activation of DA receptors on paracapsular cells or their afferents reduced their inhibitory output by a) loss of excitability as a result of an increase in a hyperpolarizing conductance, b) in case of mpcs a concomitant decreased excitatory input and c) a presynaptic effect on GABA release, shown exemplarily in lpcs.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RMP (mV)
-50
-80
10
a
b
c
PSP Amp. (mV)
0
-5
0
Time (min)
20
b
a
c
2 mV
200 ms
A2
CeA
BLA
IPSP Amp. (mV)
-10
0
*
*
cont. DA
cont. DA
A3
EPSP Amp (mV)
10
0
cont. DA
A4
RMP Change (mV)
10
0
*
*
BLA
CeA
B1
DH 10 µM
BLA
lpc
IPSC Amp. (rel.)
1
0
0
Time (min)
16
lpc
BLA
50 pA
20 ms
B2
250
DH 10 µM
IPSC Amp. (pA)
0
0
Time (min)
15
mpc
50 pA
20 pA
B3
100
IPSC Amp. (%)
0
*
*
lpc
mpc
BLA
Site of Stimulation
C1
DA 70 µM
IPSC Amp. (pA)
0
-40
0
Time (min)
20
20 pA
20 mV
50 ms
C2
*
100
Failures (%)
0
cont. DA
C3
20
IPSC Amp. (pA)
0
cont. DA

Figure 6 *D_1-receptor activation reduces inhibition from pcs.*

(A) Application of DA reduces feedforward inhibition from lpcs and mpcs and depolarizes target cells in BLA and CeA. (A1) Top trace: Resting membrane potential (RMP) of a BLA neuron vs. time. To correct for changes in the driving force the cell was later hyperpolarized using DC current injection (curr). Middle traces: EPSP (open circles) and IPSP (closed circles) amplitudes vs. time. Dopamine (DA) was applied as indicated by the horizontal line. Bottom traces: Samples of EPSP/IPSP sequences (a) before DA, (b) during application of DA and (c) after repolarization. (A2) Summary effect of DA on IPSC amplitude in the CeA and in the BLA. (A3) Summary of the effect of DA on EPSP amplitudes (data pooled from BLA and CeA). (A4) The effect of DA on RMP in the target neurons.
(B) The D_1 receptor agonist dihydrexidine (DH) depresses the output from pcs. Recordings were done in the presence of kynurenic acid (3 mM). (B1) Voltage-clamp recording from a BLA principal cell with a low chloride-containing internal solution. One stimulation electrode was placed in the BLA (open circles) and one was placed in the lpc cluster (filled circles). Stimuli were delivered to both electrodes every 10 s, a 1 s interval separated the two stimuli. Plot of the IPSC amplitude over time, normalized to baseline. DH was applied as indicated by the horizontal line. The inserts show averaged (10-20 events) traces before and after application of DH. (B2) Voltage-clamp recording from a CeA neuron with a low chloride containing internal solution, the stimulation electrode was placed in the mpc cluster. Plot of the IPSC amplitude over time with the DH application indicated by the horizontal line. The inset shows average traces (10-20 events) before and after the application of DH. (B3) Summary graph of the effects of DH on IPSC amplitude in BLA principal cells with stimulation in lpcs (lpc), in CeA neurons with stimulation in mpcs (mpc) and as a control in BLA principal cells stimulating interneurons within the BLA (BLA).
(C) The effect of DA on paired recordings between lpcs and BLA principal cells. DA depresses synaptic release from lpcs. (C1) Plot of IPSC amplitude over time in one example, DA was applied as shown by the horizontal line. The insert represents average traces (10-20 events) before (black) and after (gray) the application of DA. Top traces are the postsynaptic IPSCs, bottom traces show the presynaptic action potential. (C2) Summary of the effect of DA on the rate of failures to release despite the generation of an action potential in the presynaptic cell; in control conditions and after (DA) application of DA. (C3) The average IPSC amplitude in control condition and after (DA) the application of DA, when failures were not included in the average.

5.2. Discussion

Paracapsular cells represent a special type of interneuron in the amygdala, as they form dense cell clusters that practically ensheath the BLA complex. For one subgroup, the so-called lateral paracapsular cells (lpcs), input and output characteristics were studied in transgenic mice expressing GFP exclusively in interneurons. Furthermore, dopaminergic innervation and modulation was investigated for the entire network of paracapsular cells.

The main findings of this study are

- Lpcs receive excitatory cortical input (analogous to the medial paracapsular cells (mpcs)) and target projection cells in the BLA.
- In paired recordings, lpcs were shown to evoke IPSCs in BLA target cells with average amplitude of 16 pA and rise time and decay time constants of 5.0 ms and 46 ms respectively. This suggests dendritic rather than somatic synaptic contacts, which was confirmed by the reconstruction of a biocytin-filled lpc-principal cell pair.
- Using immunohistochemistry, particularly dense tyrosine hydroxylase positive fibers were found within lpc- and mpc clusters.
- Bath-applied dopamine (DA) caused a hyperpolarization in lpcs and mpcs of about 8 mV, resulting in substantially reduced excitability of these cells.
- This effect was mediated by a G-protein-coupled inward rectifier potassium channel (GIRK).
- D1-like receptors are responsible for the hyperpolarization observed in pcs.
- In addition to the observed hyperpolarization, we found in lpc-projection cell-pairs that DA led to an increase in the failure rate from 25 to 86%, while the amplitude of the successes was not substantially reduced (14.4 to 11.3 pA). This indicated primarily a decrease in release probability.

The pcs as a special feedforward network in the amygdala

Pcs were described morphologically nearly 20 years ago (Millhouse, 1986), however, functional data has been scarce due to their irregular location, small size and lack of any cytochemical marker. Furthermore, their spiking pattern is unusual for interneurons and rather resembles that found in BLA projection cells, making their detection in blind recordings difficult.

Indeed only the subgroup of mpcs have been further characterized (Pare et al., 2003; Royer et al., 1999), while for the lpcs physiological data, as well as input and output connectivity, have not been investigated. The present study shows that lpcs possess morphological and physiological properties similar to mpcs and, in addition, provide feedforward inhibition onto BLA neurons. These findings support the notion that the pc system as a whole can be viewed as an inhibitory feedforward network that controls the flow of information into as well as out of the BLA complex. It is tempting to speculate that a third cluster of pcs, located between the LA and BLA, might act likewise by gating signal trafficking between these two nuclei (Samson et al., 2003).

The inhibitory impact of a single lpc is small and often restricted to proximal cells

IPSCs generated by pcs are particularly small as demonstrated by paired recordings between lpcs and BLA projection neurons. An IPSC generated by a single pc would therefore exert little impact on a given target cell. Furthermore, some of our reconstructed pcs had a remarkably stubby axon, restricting their range of action to very proximal cells. This and the small IPSC they elicit raise the question as to how effective the pcs-mediated inhibition actually is at the network level. However, the dendritic tree of BLA projection cells regularly spans several 100 μm and often extends into the external capsule (Millhouse and DeOlmos, 1983). Moreover, in an acute slice preparation, axons are frequently severed by the cutting procedure.

Either spatial or temporal summation will significantly increase the inhibitory effectiveness of pcs. In fact, our data indicate connections from several lpcs to one individual BLA principal cell, allowing for spatial summation, while in-vivo recordings (Collins and Pare, 1999) revealed high spontaneous firing rates for mpcs, making temporal summation possible. At odds with this idea, however, we have not observed

any tonic activity in our preparation. In addition, pcs were not able to sustain high firing rates in response to sustained somatic current injection in contrast to other amygdala interneurons; in fact one of their main characteristics was their propensity to fatigue rapidly under prolonged current injections.

We assume that the non-physiological concentration of potassium in the ASCF used in our experiments along with the cutting might account for reduced network activity in these slices, resulting in less input to the pcs and thus less spiking activity of these cells. In addition, we work with a K gluconate intracellular solution that contains unphysiologically low levels of chloride (5 mM vs 7 to 15 mM *in vivo*), leading to a higher driving force for a chloride influx and consequently a lower resting membrane potential. Furthermore, although the rectangular current injections applied cause a marked spike frequency adaptation, a more physiological input pattern consisting of excitatory and inhibitory inputs might allow sustained firing at much higher frequencies due to the intermittent regeneration of voltage-gated conductances. In accordance with this explanation, we observed continuous firing of pcs over several minutes during enhanced network activity due to application of low doses of kainic acid.

Differences between the two subpopulations

Although the lpcs and mpcs resemble each other in many aspects, they differ in some features. In contrast to mpcs, lpcs are on average smaller and more electronically compact, forming narrower clusters with fewer cells. Royer et al., (2000) reported a lateral inhibition for mpcs with a distinct orientation along with a topographically organized excitatory input from the BLA complex, allowing for conditional gating of information by these cells. Though we found inhibitory interconnections between lpcs, there was no evidence supporting a specific orientation (data not shown) for either the lateral inhibition or the excitatory input to lpcs. The role of this lateral inhibition in lpcs thus remains speculative; it could perhaps help synchronize them and give rise to oscillatory behavior.

D1 receptors largely mediate dopaminergic modulation of the pc system

Strong evidence for a common function of pcs is provided by their specific modulation by DA and in particular by D_1 agonists. In the rat amygdala, pcs receive the densest afferents form the mesencephalic dopaminergic system with a high number of somatic and perisomatic synapses and show the highest levels of D_1-receptor immunoreactivity and D1-receptor mRNA (Asan, 1998; Fuxe et al. 2003). Our immunohistochemical results obtained in mice confirm this prominent dopaminergic innervation of pcs. A perisomatic innervation pattern should result in a direct effect on the physiological properties especially in electrotonically compact cells such as the pcs. This was indeed evidenced by the dramatic hyperpolarization and loss of excitability induced by DA and D_1 receptor agonists.

In the amygdala, such a D_1 receptor-mediated suppression appears to be restricted to pcs as BLA projection cells and other interneurons were shown to depolarize when exposed to a D_1 agonist (Kroner et al., 2005). Although DA and the D_1 receptor agonist affected all pcs in the same manner, D_2 receptor agonists depolarized only lpcs, and showed no effect in the slightly larger and less electrotonically compact mpcs. In these cells the DA-induced hyperpolarization likely depended on a synergistic effect of both D_1 and D_2 receptors as described also for pallidal neurons (Carlson et al., 1987).

Another difference involved the DAergic modulation of the excitatory input, which was suppressed onto mpcs but not onto lpcs. We lack a concise explanation for this difference and can only speculate about the reasons: selective activation of the cortical input to mpcs is difficult to achieve and therefore the response might always have been contaminated with substantial input from the BLA. The observed dopaminergic modulation might therefore be restricted to the BLA pathway for which we have not investigated an equivalent in the lpcs.

It should be noted though that dihydrexidine is only moderately selective for D_1 vs D_2 receptors (Mottola et al., 1992), and would be occupying a significant fraction of D_2-like receptors at the concentrations used in our studies. On the other hand, its functional actions at D_2 receptors are atypical, often acting as an antagonist, rather than an agonist (Darney et al., 1991; Kilts et al., 2002; Mottola et al., 2002). The pattern of effects seen in our study with dopamine, dihydrexidine, and quinpirole, and the effects of selective antagonists, are consistent with a critical role of D_1–like receptors.

DA activates GIRK channel via D1 receptors in pcs

The only ion species with a reversal potential below the resting potential of pcs in our experiments were potassium and chloride. The fact that we saw no changes in holding current of pcs patched with a CsCl-containing internal solution strongly argues in favor of a potassium current as opposed to chloride currents. We further characterized the underlying potassium conductance by voltage ramp experiments. These experiments revealed an inward rectification and a reversal potential of -84 mV, indicative of an inward rectifier potassium channel. Since experiments with the antagonists of G protein activation prevented hyperpolarization under DA, we conclude that DA activated a G protein-coupled inward rectifier potassium channel. Though these experiments were only conducted with DA, these data combined with the D1-mediated hyperpolarization led to the conclusion that D1 receptor activation results in the opening of a GIRK channel. Interestingly, a similar effect for D1 activation has been reported for GABAergic medium spiny cells (Pacheco-Cano et al., 1996) that are morphologically similar and probably ontogenetically related to pcs (Millhouse, 1986). Although there is conclusive evidence for the involvement of a GIRK channel, there might be an alternative mechanism at least for the mpcs: Recently, it was shown that D1 and D2 receptor co-activation leads to the recruitment of a novel signaling pathway involving phospholipase C-mediated calcium mobilization, which is different from the intracellular responses after stimulation of either dopamine receptor alone (Lee et al., 2004). The intracellular increase in Ca could then initiate the opening of a K_{Ca}-channel. Such a coactivation-dependent process could explain the hyperpolarization in mpcs observed under DA but also under dihydrexidine, as the concentrations we used might have co-activated D2-like receptors. It is also in accordance with the results obtained under the combined DA-D1 respectively D2-antagonist application, which did not activate a hyperpolarizing potassium current. However, Royer et al., (2000) claim, that mpcs do not posses a calcium-dependent potassium conductance.

Presynaptic effects of DA

In paired recordings between lpcs and projection cells we found an additional mechanism by which DA reduces the output of the pc network. Despite the continued

spiking of the presynaptic cell, the averaged postsynaptic response was significantly decreased mainly due to an increase in the failure rate of synaptic release. This is generally viewed as a strong indication for a reduced probability of release. In addition, the amplitude of the IPSC successes diminished from ~14 pA to 11 pA. Theoretically, one can deduce the number of synaptic contact between two cells from the pattern of the postsynaptic response, assuming a purely presynaptic effect: an exclusive increase in the failure rate of a connected pair while the amplitude remains unaltered is indicative of a single synaptic contact between these two structures. In contrast, a decrease of success amplitude indicates several synaptic contacts. The observed reduction in amplitude would thus support the notion of several synaptic contacts. However, more experiments are needed for a definite conclusion.

Pcs mediate cortical feedforward inhibition onto different amygdala nuclei

Our stimulation experiments of the external capsule, a fiber bundle composed of cortical efferents, and previous anatomical data suggest (Millhouse, 1986; Vertes 2004) that lpcs are cortically innervated, which is consistent with findings on mpcs. The latter receive robust projections from the mPFC (McDonald et al 1996) and were shown to fire upon electrical stimulation of the perirhinal area (Collins and Paré, 1999). Consistently, the decreased responsiveness of CeA output neurons following stimulation of mPFC is thought to be mediated by mpcs (Quirk et al., 2003).

In addition, Rosenkranz and Grace (Rosenkranz and Grace, 2002) found that D1-stimulation causes a potent attenuation of PFC afferent input to the BLA. However, this finding is difficult to reconcile with the excitatory effects of D1-agonists observed in interneurons in the BLA. There are two possible explanations for this discrepancy: as Rosenkranz and Grace have argued, the excitatory input to the interneurons might be reduced under D1-activation; alternatively, only a certain type of interneuron may mediate mPFC feedforward inhibition onto BLA and CeA cells and show a different modulation by DA than the interneurons within the BLA. The pcs with their prominent inhibition under DA are compelling candidates for this role. The importance of D1 receptor function on the pc network is illustrated by the fact that the disinhibitory effects of DA in the amygdala at the behavioural level are largely if not exclusively mediated by D_1 receptors. Activation of D_1 receptors by systemic application of the

selective partial D_1 agonist SKF38393 enhances both fear learning and expression (Borowski et al., 1998), whereas systemic or focal application of the D_1 receptor antagonist SCH 23390 (Greba et al., 2000; Inoue, et al., 2000) blocks either acquisition or both acquisition and expression of fear. In addition D_1 receptors play a role in other amygdala-dependent behavior, as their blockade also reduces amygdala mediated appetitive behavior (See et al., 2001).

Taking the specific pharmacology of the behavioral effects into consideration together with the fact that pcs are the only amygdala interneurons that are inactivated by D_1 receptor activation, we conclude that the pc system represents the most likely neural substrate mediating prefrontal cortical control over the amygdala and its modulation by DA.

6 General Discussion

Although the discussion of project 2 has covered several important topics concerning the pcs, there are some striking features of these cells that necessitate further debate. These conspicuous characteristics include synaptic plasticity and its impact on extinction and anxiety as well as the strong resemblance of the pc system with the nucleus reticularis thalami.

6.1 A Potential Role for the Pcs in Extinction

Due to their projection into the BLA complex and the CeA, the pcs are likely candidates for dampening multiple fear responses after extinction. This would require that some sets of inputs to pcs increase their responsiveness to the conditioned stimulus after extinction. One possible mechanism for achieving this effect involves the infralimbic region of the mPFC that projects strongly to pcs. In support of this conjecture, lesions of the infralimbic region impair extinction (Morgan et al., 2003; Quirk et al., 2000) and electrical stimulation of the infralimbic region reduces the expression of conditioned fear (Milad and Quirk, 2002). Interestingly, neurons of the infralimbic region do not respond to tones during the first extinction session but respond robustly 24 hours later, when rats are recalling extinction (Milad and Quirk, 2002), suggesting a role for the mPFC not in the initial learning of extinction but in the consolidation and subsequent recall of extinction memory. Thus, the mPFC might be a site, where extinction memory is stored.

The pcs could participate in this task as it was recently reported that at least basolateral inputs into the medial cluster of pcs exhibit NMDA-dependent LTP and LTD (Royer and Pare, 2002). Further support for this hypothesis comes from the fact that NMDA antagonists given systemically (Santini et al., 2001) or directly into the amygdala (Lin et al., 2003) prevent long-term memory from extinction. Experimental evidence is currently lacking as to whether mPFC inputs to pcs are indeed modifiable. One of the experimental difficulties in addressing this question lies in the specific activation of

cortical/mPFC synapses onto pcs, as these cells are also innervated by basolateral afferents.

The mechanisms underlying extinction are of particular interest because of its implication for anxiety disorders. As suggested by LeDoux (1996) and Morgan et al., (1993), insufficient inhibition of the amygdala by the mPFC could predispose an individual to develop anxiety disorders. In fact, recent neuroimaging studies of patients with posttraumatic stress disorder (PTSD) show decreased activity in medial prefrontal/anterior cingulate areas, correlated with increased activity in the amygdala (Bremner et al., 1999; Shin et al., 2004). Given that extinction is the basis for exposure therapy for PTSD (Bouton, 1988), an obvious therapeutic strategy should be to strengthen extinction consolidation. Recent experiments in rats suggest that extinction can be facilitated with intra-amygdala infusion of D-cycloserine, a glycine site agonist of NMDA receptors (Ledgerwood et al., 2004). Pcs in the amygdala are a likely site of action of D-cycloserine, which may facilitate potentiation of prefrontal or basolateral inputs.

6.2. Analogy Between the Pc System and the Nucleus Reticularis Thalami

In terms of its location and connectivity the pc network strikingly resembles another inhibitory network of the brain, the nucleus reticularis thalami (nrt). The nrt, a thin layer composed entirely of GABAergic inhibitory neurons (Houser et al., 1980; Spreafico et al., 1991), receives input from pyramidal cortical neurons and excitatory relay cells of the ventrobasal complex of the thalamus (Pinault and Deschenes, 1998), while projecting to the latter. By providing inhibitory input to thalamic relay cells, the neurons of the nrt generate synchronized activity during sleep and seizures (Bal et al., 1995; von Krosigk et al., 1993). Furthermore, by virtue of its anatomy and physiological properties, the nrt is in an ideal position to gate the flow of information between thalamus and cortex and consequently a “sensory gate-keeper” function has been implicated in higher cognitive processes e.g. attention (Guillery et al., 1998; McAlonan et al., 2000).

While for the medial pc cluster a dual innervation (analogous to the one observed in nrt cells) involving cortical as well as basolateral afferents has been shown (Royer et al., 1999; Vertes 2004; Sesack et al., 1989), such functional data are lacking for the lateral cluster. There are anatomical hints for an equally twofold innervation of these neurons, though. As described by Millhouse (1986), axon collaterals of basolateral projection cells appear to terminate among cells of both pc clusters, the medial and the lateral cluster. Furthermore, extracellular stimulation of the BLA complex generates EPSPs in cells of the lateral pc cluster, as observed in preliminary experiments performed in our lab. These BLA-lateral pc connections could be further investigated by recording from pcs while stimulating BLA activity by focal application of glutamate, thus ruling out the activation of passing fibres.

One of the key features of the nrt is its capacity to generate rhythmic activity and to entrain the connected thalamic nuclei with these rhythms. Substantial inter-connections between the nrt cells are necessary for the network to synchronize (Pinault and Deschenes, 1998) and to impose rhythmic activity on thalamic neurons. Furthermore, low voltage-activated calcium channels (T-type), which can produce burst firing, are the most important cellular features of nrt cells underlieing these rhythms (Bal et al 1995). Consistent with results obtained by the group of Paré (Royer et al 1999, 2000) for the medial pc cluster, we also detected strong interconnections – or lateral inhibition – in the pc cluster along the external capsule. This was evidenced by paired recordings of pcs (unpublished data).

In further experiments data on rhythmic activity within the pc network will have to be obtained and the underlying mechanisms will have to be elucidated. Since individual pcs can be easily patched, the existence of T-type calcium channels and their possible implication in burst firing of pcs can be studied.

There is a general agreement that GABA-mediated inhibitory synapses are the mechanism by which neurons within the nrt network interact. In addition to the classical chemical synapses, nrt cells also appear to network via electrical synapses (Landisman et al., 2002). As recent work on GABAergic neurons in the neocortex (Galarreta and Hestrin, 1999) and other parts of the mammalian brain (Galarreta and Hestrin, 2001) suggests, electrical coupling may be a common feature of inhibitory networks, helping to synchronize such large assemblies of interneurons. In line with this notion, EM pictures of pcs show dense packing of these cells along with substantial somatic appositions between neighbouring cells (Asan et al 1998). These findings are strongly

suggestive of gap-junction-mediated connections and cjould be confirmed by paired recordings from pcs.

A rhythmically active interconnected inhibitory network could serve an important function in controlling the association of different sensory stimuli into a single event. During rhythmic synchronized activity the area targeted by the pcs would oscillate between a permissive and a non-permissive state. Input from different sensory areas arriving simultaneously during a permissive episode would have a much higher chance of triggering a fear reaction or induce fear learning than input that was more dispersed in time. DA by reducing excitability and inhibitory output of pcs most likely would also profoundly affect their rhythmic behavior. The de-synchronized and inactivated pcs would allow association of sensory information over a larger time window.

6.3. The PC System May Play a Crucial Role in Reinstatment of Drug-seeking Behaviour

The amygdala is generally known as one of the brain regions where addiction drugs act during the rush/euphoria phase (Breiter and Rosen, 1999). In particular, the basolateral amygdala is implicated in reward association and drug-seeking, as lesions of the basolateral amygdala reduce cocaine-seeking behaviour elicited or maintained by cocaine-related stimuli, prevent conditioned opiate withdrawal responses and impair conditioned behaviour maintained by stimuli associated with sexual reward. Furthermore, this nucleus appears to be important in craving, evidenced by studies showing that cue-induced cocaine craving in humans is associated with increased neural activation in this area (measured as increase in blood-flow using positron emission tomography) (Childress et al 1999) and that cocaine cues increase dopamine release within this nucleus in the rat (Weiss et al 2000).

Based on these findings, Ciccocioppo et al. (2001) examined whether a drug-predictive stimulus retains its efficacy to induce cocaine-seeking behavior after long-term abstinence and the neurobiological basis of this phenomenon. They trained rats to associate a certain tone with the availability of intravenously administered cocaine (versus a light signal associated with saline) and then placed the animals on extinction conditions (the tone was given without the availability of cocaine until the rat

exhibited no longer a response to it). Even after extinction training and four month of absence, the presentation of the tone triggered strong drug-seeking behaviour, documenting that the motivating actions of environmental cues acting as discriminative stimuli for drug availability can remain intact over a substantial length of time. Strikingly, the selective dopamine D1 antagonist SCH 39166, administered before the presentation of the tone, reversed the increases in cocaine-seeking behaviour: The rats behaved as if they were exposed to the light signal, the stimulus associated with the non-rewarding saline.

Furthermore, Fos protein expression was measured to determine the specific brain areas involved. In rats presented with the tone, the number of Fos-immunoreactive neurons selectively increased within the basolateral amygdala and medial prefrontal cortex. Again, the D1 receptor antagonist SCH 39166 reversed this effect.

Taking into consideration the specific action of D1 receptor agonists on paracapsular cells, it seems reasonable to assume a crucial involvement of these cells in this process. Indeed, the findings by Ciccocioppo et al. (2000) parallel data by Rosenkranz and Grace (2002) describing a D1-receptor-mediated attenuation of cortical inhibitory control and disinhibition of the amygdala. We postulated that the paracapsular cell system contributes significantly to the observed disinhibition and that they probably do so as well in cue-induced reinstatment of cocaine-seeking. While in situations related to fear the reduced inhibitory activity of the paracapsular cells allows for activation of CeA neurons by BLA cells, thereby triggering the typical fear reaction, the CeA is most likely not the relevant output nucleus in reinstatement. A better candidate would be the nucleus accumbens, as the BLA complex maintains strong (unilateral) projections to this area of the brain (Aggleton, 2000) and direct interactions between the BLA and nucleus accumbens core were demonstrated to underlie cocaine-seeking behaviour in rats (Di Ciano and Everitt, 2004). The latter study also emphasized the activation of excitatory receptors (AMPA or kainate receptors) on cells of the nucleus accumbens, pointing to a glutamatergic projection from BLA, which is in accordance with anatomical findings (Weiss et al., 2000)

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8 Abbreviations

AB	accessory basal nucleus of the amygdala
AMPA	amino-3-hydroxy-5-methyl-4-isoxazol-propionic acid
B	basal nucleus of the amygdala
BLA	basolateral complex of the amygdala, comprising LA and B
BZ	benzodiazepine
CB1	cannabinoid receptor 1
CCK	cholecystokinin
CeA	central nucleus of the amygdala
CeL	lateral part of the CeA
CeM	medial part of the CeA
CS	conditioned stimulus
DA	dopamine
DAB	diaminobenzidine
EPSC	Excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
IPSP	inhibitory postsynaptic potential
IPSC	inhibitory postsynaptic current
GABA	gamma-amino-butyric acid
GAD	glutamic acid decarboxylase
GFP	green fluorescent protein
GIRK	G protein coupled inward rectifier potassium
GluR (1 or 2)	glutamate receptor subunit 1 or 2
LA	lateral nucleus of the amygdala
LTP	long-term potentiation
lpcs	lateral (cluster of) paracapsular cells
mpcs	medial (cluster of) paracapsular cells
mIPSC	miniature inhibitory postsynatpci currents (“minis”)
mPFC	medial prefrontal cortex
NMDA	N-methyl-D-aspartate
PB	phosphate buffer

PBS	phosphate-buffered saline
pc(s)	paracapsular cell(s)
PFC	prefrontal cortex
PV	parvalbumin
TPMPA	1,2,5,6-tetrahydropyridine-4-yl-methylphosphinic acid
TTX	tetrodotoxin
Tris	tris(hydroxymethyl)aminomethane
VTA	ventral tegmental area
US	unconditioned stimulus

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